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(71) Applicant: **TARGOVAX ASA** [NO/NO]; Lilleakerveien 2C, 0283 Oslo (NO).

(72) Inventors: **ERIKSEN, Jon Amund**; c/o Targovax ASA, Lilleakerveien 2C, 0283 Oslo (NO). **JONSBU, Einar**; c/o Targovax ASA, Lilleakerveien 2C, 0283 Oslo (NO). **IVERSEN, Berit**; c/o Targovax ASA, Lilleakerveien 2C, 0283 Oslo (NO).

(74) Agent: **ARENDS, William Gerrit**; Marks & Clerk LLP, 15 Fetter Lane, London EC4A 1BW (GB).

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(57) Abstract: There is disclosed a formulation comprising a GM-CSF protein having at least 60% sequence identity to the polypeptide sequence of SEQ ID NO: 2. The formulation comprises a stabiliser, wherein the stabiliser comprises a poloxamer present in an amount of 0.5-3.0wt%, excluding water. The formulation does not comprise more than 0.2wt%of human serum albumin or recombinant human albumin.

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A Formulation

Field of Invention

The present invention provides formulations comprising a GM-CSF protein and the use of the formulations as a medicament, particularly in the treatment of cancer.

Background

Granulocyte macrophage colony-stimulating factor (GM-CSF) is monomeric glycoprotein secreted by macrophages, T-cell, mast cell, natural killer T-cells, endothelial cells and fibroblasts. It functions as a cytokine, and is an immune system stimulator and hematopoietic growth factor. GM-CSF is produced as a response to immune or inflammatory stimuli and plays an important role in regulating the proliferation, differentiation, survival and activation of hematopoietic cells such as granulocytes and monocytes, neutrophils, basophils and eosinophils, erythroid cells, megakaryocytes and T cells. In particular, GM-CSF stimulates stem cells to produce granulocytes and monocytes, as well as having effects on mature cells of the immune system, such as inhibiting neutrophil migration. GM-CSF has also been shown to be involved in the maturation, mobilisation and antigen presentation of myeloid dendritic cells (DCs), *in vivo* or *ex vivo*. For example, Arellano and Lonial (Biologics: Targets and Therapy, 2008:2(1), 13-27, "Clinical uses of GM-CSF: a critical appraisal and update") and van de Laar *et al*. (Blood, 12 April 2012:119(15), "Regulation of dendritic cell development by GM-CSF; molecular control and implications for immune homeostasis and therapy") have shown that, when injected under the skin, GM-CSF attracts and activates the maturation process of local dendritic cells (DCs), by binding to the GM-CSF receptor on DCs, to become functionally active antigen presenting cells (APCs). GM-CSF, thus, promotes Th1 immune responses, cytotoxicity, anti-angiogenesis as well as allergic inflammation, and the development of autoimmunity.

Naturally-occurring, mature, wild-type, human GM-CSF is a 127 amino acid residue-protein, consisting of amino acid residues 18-144 of the sequence shown in UniProtKB data base entry P04141 (http://www.uniprot.org/uniprot/P04141) and SEQ ID NO: 1. The amino acid sequence of mature, wild-type human GM-CSF is shown in SEQ ID NO: 2. Amino acid residues 1-17 of the sequence shown in UniProtKB data base entry P04141 and SEQ ID NO: 1 are a signal sequence, and do not form part of the mature GM-CSF product. Mature wild-type human GM-CSF is glycosylated at positions 22, 24, 26, 27, 44 and 54 of the amino acid sequence of SEQ ID NO: 1, which corresponds to amino acid residues 5, 7, 9, 10, 27 and 37 of the amino acid sequence of SEQ ID NO: 2.

Pharmaceutical formulations of GM-CSF have been developed for raising white blood cell counts and boosting the immune system after chemotherapy, for mobilisation of hematopoietic progenitor cells and myeloid reconstitution after transplantation of autologous peripheral blood progenitor cells, and for myeloid reconstitution after autologous or allogenic bone marrow transplantation. Pharmaceutical formulations of GM-CSF are also used for improving the immune response to peptide vaccines. In particular, the peptides in a peptide vaccine must be presented on dendritic cells in order to have an effect, and GM-CSF attracts dendritic cells. A further use of pharmaceutical formulations of GM-CSF is the acceleration of myeloid recovery following bone marrow transplantation.

Molgramostim is GM-CSF produced by *E. coil.* The resulting GM-CSF product has 128 amino acid residues, has a molecular weight of approximately 14,600, is non-glycosylated and has an additional N-terminal methionine which is only partially cleaved (i.e. the N-terminal methionine is cleaved from some but not all molecules). The amino acid sequence of Molgramostim is shown in SEQ ID NO: 3. One existing commercial formulation of Molgramostim (rHuGM-CSF) contains, before lyophilisation, 0.15mg/ml (targeted value of $1.64x10^6$ IU/ml) Molgramostim, 50mg/ml mannitol, 19.4mg/ml Magrocol 4000, 1mg/ml human serum albumin (HSA), 0.99mg/ml disodium phosphate and 0.42mg/ml potassium dihydrogen phosphate.

Sargramostim is GM-CSF produced by yeast, and has the amino acid sequence shown in SEQ ID NO: 4. Sargramostim has an amino acid substitution at position 23 of the amino acid sequence of mature wild-type human GM-CSF (SEQ ID NO: 2), replacing arginine with leucine. Due to being produced by yeast, Sargramostim is hyper-glycosylated as compared to mature wild-type human GM-CSF, by about 100-150 times. In particular, Sargramostim consists of three molecular species having relative molecular weights of 19,500, 16,800 and 15,500, due to the different levels of glycosylation, and these forms are present in an amount of 25-42%, 14-32% and 35-53%, in order of elution (U.S. Pharmacopeia; Sargramostim). This hyper-glycosylation affects the potency of GM-CSF, with the potency of Sargramostim being about 50% (mol/mol) of the potency of Molgramostim. This difference is disproportionately large, considering that the average molecular weight of Sargramostim is only 20% more than the molecular weight of Molgramostim. Hyper-glycosylation can also change the biological activity by changing the 3D structure of the protein, and can affect the immunogenicity of GM-CSF.

Sargramostim is marketed in the USA as Leukine® (approved by the FDA in March 1995), which contains 40mg/ml mannitol, 10mg/ml sucrose and 1.2mg/ml tromethanine, and is available in either liquid or lyophilised form. The liquid form of Leukine® contains 500µg

($2.8x10^6$ IU) Sargramostim and 1.1% benzyl alcohol in a 1ml solution, and has a pH of 6.7-7.7. The lyophilised form contains 250µg ($1.4x10^6$ IU) Sargramostim per vial, and has a pH of 7.1-7.7.

Although GM-CSF has several pharmaceutical uses, there is currently no approved formulation of GM-CSF available on the European market. Leukine® has been used off-label, in Europe, for clinical studies, as an immune-stimulator for cancer vaccines.

Some current commercial pharmaceutical formulations of human GM-CSF use human serum albumin (HSA) or recombinant human albumin (rHA) as a stabiliser. HSA is a serum albumin found in human blood, and produced by the liver. The amino acid sequence of immature HSA is shown in SEQ ID NO: 5, wherein amino acid residues 1-18 are a signal sequence, amino acid residues 19-24 are a pro-peptide, and amino acid residues 25-609 forms the mature protein. It has been used in therapeutic applications to replace lost fluid (such as after blood loss), as well as a pharmaceutical excipient. However, the only source of human serum albumin is fractionation of donated blood, which involves possible contamination by viruses and prions (Chuang and Otagiri; Drugs Today (Barc)., 2007;43(8):547-561), such as those causing hepatitis and HIV (www.albumin.prg/clinical-use-of-albumin).

An alternative to HSA is recombinant human albumin (rHA), which is produced by yeast, bacteria or plant-based expression systems. rHA has the same amino acid sequence as HSA (shown in SEQ ID NO: 5), but may have a different glycosylation pattern from HSA. As yeast and plants are animal-free, rHA do not present risks commonly associated with animal-derived products (www.albuminbio.com/safety). On the other hand, the quality of rHA differs depending on the source, due to the difference in formation of dimers and multimers.

However, both HSA and rHA are no longer desirable in pharmaceutical formulations. It has been found that both HSA and rHA form as monomers, dimers and multimers, which are undesirable in pharmaceutical compositions, and mask impurities in the formulations, such that the purity measurements of existing formulations may not be accurate. HSA and rHA may even cause some of these impurities. As a result of HSA and rHA masking impurities in the formulation, it is difficult to control the drug product, in terms of a safe biological dose, control of degradation products and control of purity.

HSA is also undesirable from a regulatory point of view. In particular, as it is of human origin, from human donors, HSA is difficult to produce and human donors result in contaminants being

present in the samples. The use of human donors also means that HSA must be certified BSA and TSA-free, which requires a lot of testing. Furthermore, the use of human donors causes variance in potency between batches of HSA. Moreover, regulatory agencies are restricting the use of HSA because of the concerns about potential infectious agents in animal-derived products (Ohtake *et al.*, "Interactions of formulation excipients with proteins in solution and in the dried state", Adv. Drug Deliv. Rev. (2011), doi:10.1016/j.addr.2011.06.011).

With regard to Sargramostim, the glycosylation of GM-CSF means that the molecular weight of the drug is 20% greater than the molecular weight of Molgramostim, such that, as mentioned above, Sargramostim has only half the potency of Molgramostim, mol/mol. This means that twice the amount of Sargramostim is required for the same therapeutic effect. Thus, treatment with Sargramostim is more expensive than with Molgramostim (because a greater amount of Sargramostim is required for the same effect). In addition, treatment with Sargramostim is less convenient than with Molgramostim, because a longer interval is required between administration of GM-CSF and a subsequent pharmaceutical, such as a vaccine, in order to allow the blister created by Sargramostim to reduce in size, and because injection of the greater volume of Sargramostim is more painful than injection of Molgramostim. It is also not known whether a solution of Sargramostim, having double the volume but only half the potency of a corresponding solution of Molgramostim, has the same effect *in vivo* as a corresponding solution of Molgramostim. Furthermore, yeast are more difficult to use, to express proteins such as GM-CSF, than *E. coli*, and the use of yeast produces a risk of severe allergic reactions and anaphylaxis in patients having a hypersensitivity to yeast-derived products.

Treatment using pharmaceutical formulations of GM-CSF with peptide vaccines requires intradermal injection of the formulation. This creates a blister or bleb, and the peptide vaccine is administered to the same site after a period of about 20 minutes. This waiting time allows the recruitment of dendritic cells to the injection site, in order to ensure an immune response, and allows the blister to reduce in size somewhat before the peptide vaccine is injected. If the blister is not allowed to recede, then the subsequent injection of the vaccine into the same site can be very painful for the subject, and, in addition, may result in leakage from the blister. It is considered amongst some in the art that the reduction of the blister to avoid pain is a more important reason for the interval between injections of the GM-CSF formulation and the peptide vaccine than the recruitment of dendritic cells.

Molgramostim and Sargramostim have now been replaced in their role in post-chemotherapy treatment (i.e. raising white blood cell counts) by granulocyte-colony stimulating factor (G-CSF;

e.g. Amgen). For example, although Leukine® is still on the market in the US, it is a marginal product compared to G-CSF products such as Amgen's Neulasta® (pegylated G-CSF; pegfilgrastim). G-CSF stimulates bone marrow to produce granulocytes and stem cells, and release them into the blood stream. G-CSF also stimulates the survival, proliferation, differentiation and function of neutrophil precursors and mature neutrophils.

However, it is doubtful that G-CSF would be suitable for promoting the presentation of peptide vaccines by dendritic cells. In particular, G-CSF and GM-CSF target, and have significantly different effects on the mobilisation and differentiation of, different subsets of dendritic cells (DC). GM-CSF preferentially enhances the activity and numbers of type-1 DCs (DC1), which is the subset responsible for skewing the immune system towards Th1-type and cytotoxic T-cell responses. This increase in DC1 content and activity, following local administration of GM-CSF, supports a role for GM-CSF as an immune stimulant and vaccine adjuvant in cancer patients (Arellano & Lonial; Biologics: Targets and Therapy, 2008:2(1), 13-27, "Clinical uses of GM-CSF: a critical appraisal and update"). In contrast, G-CSF preferentially enhances the differentiation of type-2 DCs (DC2), which do not induce an immune response.

Thus, there is a need to provide an alternative pharmaceutical formulation of GM-CSF which overcomes the problems and disadvantages discussed above. In particular, it is desirable to provide a formulation of GM-CSF that overcomes the problems of inaccurate purity measurements, batch-variation in potency, and that has good potency, purity and stability. It is desirable to provide a formulation of GM-CSF that is safer, of better quality and more controllable than previous and existing formulations.

Summary of Invention

The present invention provides solutions to the problems discussed above because it has now, surprisingly, been found that formulations of a GM-CSF protein, wherein the formulations contain no or substantially no HSA or rHA, have at least the same potency as previous formulations. It has been found that the formulations of the present invention are more controllable, of better quality and are safer than previous formulations, because the purity of the formulations of the invention can be tested more accurately. The formulations of the invention have good potency and stability, and have improved purity.

Thus, in a first aspect of the invention, there is provided a formulation comprising a GM-CSF protein having at least 60% sequence identity to the polypeptide sequence of SEQ ID NO: 2 and a stabiliser comprising a polymer or a surfactant.

Preferably, the formulation substantially does not comprise and human serum albumin or recombinant human albumin, for example, no more than 0.2wt% of human serum albumin or recombinant human albumin.

Preferably, the the GM-CSF protein has at least 70%, 80%, 90%, 95% or 99% sequence identity to the polypeptide sequence of SEQ ID NO: 2.

Advantageously, the stabiliser further comprises a disaccharide or arginine.

Conveniently, the disaccharide or arginine is present in an amount of 10.0-99.0wt%, preferably 30.0-99.0wt%, most preferably 95.0-98.0wt%, excluding water.

Preferably, the disaccharide is sucrose and/or mannitol.

Advantageously, the polymer or the surfactant is present in an amount of 0.5-3.0wt%, preferably 1.0-2.5wt%, excluding water.

Preferably, the polymer is a poloxamer, more preferably P188.

Conveniently, the surfactant is polysorbate 20 or polysorbate 80.

Advantageously, the formulation further comprises a buffer.

Conveniently, the buffer is present in an amount of 0.5-5.0wt%, preferably 1.0-2.5wt%, excluding water.

Preferably, the buffer is potassium phosphate.

Preferably, the formulation does not comprise more than 0.02wt% of human serum albumin or recombinant human albumin.

Advanatgeously, the GM-CSF protein is present in an amount of 0.001-10.0wt%, preferably 0.01-10.0wt%, most preferably 0.1-10.0wt%, excluding water.

Conveniently, the GM-CSF protein has at least 60%, 70%, 80%, 90%, 95%, 99% or 100% sequence identity to SEQ ID NO: 3.

Conveniently, no more than 25% of the amino acid residues of the GM-CSF protein are glycosylated.

Preferably, no more than 5% of the amino acid residues of the GM-CSF protein are glycosylated.

Advantageously, the formulation is liquid.

Preferably, the formulation has a pH in the range of 6.5 to 9.0.

Advantageously, the formulation has a pH in the range of 7.3 to 8.1, preferably wherein the formulation has a pH of 7.3 or 8.1, and more preferably a pH of 8.1.

Conveniently, the formulation is a frozen liquid.

Preferably, the formulation comprises water in an amount of 10.0-99.0wt%, preferably 50.0-99.0wt%, and most preferably 90.0-99.9wt%.

Alternatively, the formulation is freeze dried and contains less than 5% residual water.

Preferably, the formulation has a potency in the range of $0.26x10^6$ to $8.33x10^6$ IU, preferably in the range of $0.26x10^6$ to $0.42x10^6$ IU.

Conveniently, the formulation has a purity of at least 95% when measured after 12 weeks of storage, preferably at least 97% when measures after 12 weeks of storage.

Advanageously, the formulation is for use as a medicament.

Preferably, the formulation is for use simultaneously or sequentially with a peptide or protein vaccine.

Definitions

The percentage "identity" between two sequences may be determined using the BLASTP algorithm version 2.2.2 (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", nucleic Acids Res. 25:3389-3402) using default parameters. In particular, the BLAST algorithm can be accessed on the internet using the URL http://www.ncbi.nlm.nih.gov/blast/.

"GM-CSF protein", as used herein, is, in some embodiments, a protein having at least 60% sequence identity to the mature monomeric glycoprotein having the amino acid sequence of SEQ ID NO: 2. Naturally-produced, wild-type GM-CSF is secreted by macrophages, T-cell, mast cell, natural killer T-cells, endothelial cells and fibroblasts, and functions as a cytokine and a hematopoietic growth factor, to stimulate the immune system. As mentioned above, the amino acid sequence of naturally-produced, wild-type, human GM-CSF consists of amino acid residues 18-144 of UniProtKB database entry P04141 (i.e. SEQ ID NO: 1), and is shown in SEQ ID NO: 2. Mature, wild-type human GM-CSF is glycosylated at amino acid residues 22, 23, 26, 27, 44 and 54. In some embodiments, a GM-CSF protein corresponds to the mature monomeric glycoprotein having the amino acid sequence of SEQ ID NO: 2. In order to be considered to be GM-CSF, a GM-CSF protein must have at least 1% of the cytokine activity of mature, wild-type GM-CSF (i.e. SEQ ID NO: 2).

"Cytokine activity", as used herein, means that a protein is capable of affecting the differentiation and behaviour of other, nearby, cells. Referring specifically to GM-CSF, the term "cytokine activity" means that GM-CSF is capable of attracting and activating the maturation process of local dendritic cells (DCs) into mature antigen-presenting cells (APCs). GM-CSF functions as a cytokine by binding to the GM-CSF receptor in the DCs (Arellano and Lonial, Biologics: Targets and Therapy, 2008:2(1), 13-27, "Clinical uses of GM-CSF: a critical appraisal and update"; and van de Laar *et al.*, Blood, 12 April 2012:119(15), "Regulation of dendritic cell development by GM-CSF; molecular control and implications for immune homeostasis and therapy"). A suitable assay for determining the cytokine activity of a protein is an *in vitro* bioassay as described in "European Pharmacopeia 7.0, 01/2008:1641; Monograph of Molgramostim Concentrated Solution". In particular, this assay involves stimulating TF-1 cells with GM-CSF, and subsequently measuring the conversion of stains such as tetrazolium bromide (MTT). MTT is converted to purple formazan, which is measured spectrophotometrically. In order to be considered to have cytokine activity, a GM-CSF protein must have a minimum activity 0.7×10^5 IU/mg, as measured by the above-mentioned bioassay in

"European Pharmacopeia 7.0, 01/2008:1641; Monograph of Molgramostim Concentrated Solution".

A "stabiliser", as used herein, is a molecule used to stabilise proteins by maintaining protein structure, integrity and biological activity, to suppress protein aggregates, to prevent degradation, to reduce surface adsorption or to provide physiological osmolality. Stabilisers are well-known in the art and include sugars, salt, polymers, surfactants and amino acids.

A "surfactant", as used herein, is a compound which lowers the surface tension between two liquids, or a liquid and a solid. Surfactants are well known in the art, and include, for example, polysorbate 20 (P20), polysorbate 80 (P80), N-octyl glucoside, Pluronic, Brij 35, Brig 30, Triton X-10, and sodium dodecyl sulfate (SDS), amongst others.

A "poloxamer", as used herein, refers to a non-ionic co-polymer of polyoxypropylene and polyoxyethylene. When using the generic term "poloxamer", these co-polymers are named with a "P" (for poloxamer) followed by a three-digit number, wherein the first two digits multiplied by 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit multiplied by 10 gives the percentage polyoxyethylene content. For example, the name P188 indicates that the poloxamer has an approximate molecular mass of 1800g/mol, and a 80% polyoxyethylene content.

"Human serum albumin", as used herein, is the most abundant protein found in human plasma. It is a soluble and monomeric protein, and is produced by the liver. It is produced by fractionating human plasma, and is used as a stabiliser for drugs and vaccines, as well as in imaging and cell culture, and therapeutic uses. Immature wild-type HSA has the amino acid sequence shown in SEQ ID NO: 5.

"Recombinant human albumin", as used herein, is human serum albumin which has been produced by genetic recombination, typically using *Saccharomyces cerevisiae.* Recombinant human albumin has the same amino acid sequence as human serum albumin (SEQ ID NO: 5).

"Potency", as used herein, is a measure of biological efficacy and the amount of drug required to produce an effect of a given intensity, and is measured in International Units (IU). In particular, the potency of GM-CSF is tested by incubating TF-1 cells with varying dilutions of test and reference preparations of GM-CSF, and then incubating the TF-1 cells with a solution of MTT. MTT is converted to purple formazan by the TF-1 cells, which is measured

spectrophotometrically, and compared to dilutions of the appropriate International Standard of GM-CSF as defined by the WHO.

Brief Description of the Figures

Figure 1 is a schematic illustration of a binary CG-MALS experiment, showing the concentrations in each gradient step of sample 1 (AUX-I) and sample 2 (AUX-II).

Figure 2 is an exemplary thermogram of the nanoDSC measurement of variant #1 of the pre-screening with different buffering agents.

Figure 3 is a diagram showing the main effect for A2 from the first DoE matrix.

Figure 4 is a diagram showing the main effect for T_{onset} from the first DoE matrix.

Figure 5 is a photograph of sub-variant #2 (PS20, N_2) after light stress.

Figure 6 is a representative SE-HPLC chromatogram of 6 µL "control" at T=0 by SE-HPLC (Acquity column 3.2.2) (A), and an enlargement of part of the chromatogram (B). GM-CSF monomer: RT = 5.4 min. Human serum albumin (HSA) monomer (RT = 4.5 min), dimer (RT = 3.9 min), and multimer (RT ~ 3.7 min). Measurement at 214 nm.

Figure 7 is two representative SE-HPLC chromatograms (Superdex column 3.2.3) of light stressed samples of test variant #2 with PS20 and N2 (A), and with rHA and N2 (B). The last peak at around RT=42 min is caused by L-arginine. Monomeric GM-CSF: 33.2 min. Monomeric rHA: 28 min. Measurement at 214 nm.

Figure 8 is a graph showing the minimum purity of test variants, by showing the minimum relative GM-CSF areas cumulative, throughout all stress conditions determined by SE-HPLC. Peaks of rHA and human serum albumin ("control"), in rHA / HSA containing samples, were not integrated.

Figure 9 is an overlay of IEX-HPLC chromatograms of injections of 15 µL GM-CSF working standard (1 mg/mL) throughout the entire forced degradation study. Chromatograms are in chronological order starting at the black chromatogram at the bottom (beginning of FD study) and going up to the dark green one at the top (end of FD study).

Figure 10 is a representative IEX-HPLC chromatogram of 15 µL of a human serum albumin containing sample (variant "control"). Measurement at 214 nm.

Figure 11 is two representative chromatograms of test variant #2 by RP-HPLC. A: test variant #2 with PS20 and air after light stress; B: test variant #2 with rHA and air after light stress. Measurement at 214 nm. GM-CSF monomer (RT = 25.4 min), rHA monomer peak (RT ~ 16.5 min).

Figure 12 is a graph showing the minimum purity, by showing the relative GM-CSF areas, throughout all stress conditions determined by RP-HPLC. Monomeric peaks of rHA and human serum albumin (HSA) ("control") were not integrated.

Figure 13 is an overview of the measurement principle of the DynaPro Plate reader II (dynamic light scattering).

Figure 14 is a schematic illustration of the position of a thermocouple inside a vial. The dot close to the base of the vial displays the point where the temperature was measured. The position of the sensor (dot) between vial bottom and fill surface might vary from vial to vial.

Figure 15 is exemplary photographs of representative vials after the GM-CSF drug product lyophilization feasibility test run. Vials are shown from side view (upper row) and bottom view (lower row) in comparison to current lyophilized drug product #C ("control").

Figure 16 is an SE-HPLC chromatogram recorded using 100µL of 0.3mg/mL GM-CSF Control Sample 1 diluted with running buffer (A), and an enlargement of the chromatogram (B). Measurement at 214nm. GM-CSF main peak at 33 minutes retention time, fragments detectable at 38 minutes retention time.

Figure 17 ia a SE-HPLC chromatogram recorded using 100µL of 0.3mg/mL final formulation variant #1 (representative for all final formulation variants #1, #2, #1d, #2d), measured at 214nm (A), and an enlargement of the chromatogram (B). GM-CSF main peak at 33 minutes retention time, fragments detectable at 38.7 minutes retention time, aggregates detectable at 28.1 minutes.

Figure 18 is a SE-HPLC chromatogram recorded using 100µL of 0.3mg/mL of lyophilized existing commercial formulation ("control" (#C)), measured at 214nm (A), and an enlargement of the chromatogram (B). GM-CSF main peak at 33 minutes retention time, aggregates detectable at 22.7 minutes retention time, HSA peak at 26.6 minutes.

Figure 19 is a RP-HPLC chromatogram recorded using 100 µL of 0.3 mg/mL GM-CSF Control Sample 2 diluted with dilution buffer (A), and an enlargement of the chromatogram (B). Measurement at 214 nm. GM-CSF main peak at 25.2 minutes retention time.

Figure 20 is a RP-HPLC chromatogram recorded using 100 µL of 0.3 mg/mL GM-CSF drug product variant #1 (representative for all variants #1, #2, #1d, #2d), measured at 214 nm (A), and an enlargement of the chromatogram (B). GM-CSF main peak at 25.2 minutes retention time.

Figure 21 is a RP-HPLC chromatogram recorded using 100 µL of 0.3 mg/mL lyophilized existing commercial formulation ("control" (#C)), measured at 214 nm (A), and an enlargement of the cromatogram (B). GM-CSF main peak at 25.3 minutes retention time, HSA peak at 16.1 minutes.

Figure 22 is a chromatogram of GM-CSF Control Sample 3 generated positive control showing the recorded UV-signal (continuous line) as well as the weight average molecular weight by MALS (dots).

Figure 23 is an overlay of SE-HPLC chromatograms of the three different GM-CSF Control Samples (A), and an enlargement of the overlay (B). Measurement at 214 nm.

Figure 24 is an overlay of SE-HPLC chromatograms of GM-CSF Control Sample 3 and an existing commercial formulation of Molgramostim (A), and an enlargement of the overlay (B). Both measurements used the drug substance (i.e. not formulated). Measurement at 214 nm.

Figure 25 is an overlay of SE-HPLC chromatograms of GM-CSF Control Sample 3 and lyophilizates of drug products produced in Example 5 (A), and an enlargement of the overlay (B). Measurement at 214 nm.

Figure 26 is an overlay of SE-HPLC chromatograms of final formulation variant #1 drug product produced in Example 5 to its corresponding placebo solution without GM-CSF drug substance (A), and an enlargement of the overlay (B). Measurement at 214nm.

Figure 27 is an overlay of SE-HPLC chromatograms of final formulation variant #2 produced in Example 5 to its corresponding placebo solution without GM-CSF drug substance (A), and an enlargement of the overlay (B). Measurement at 214nm.

Figure 28 is an overlay of SE-HPLC chromatograms of GM-CSF Control Sample 3 and a positive control stressed at 55 °C for 60 hours (A), and an enlargement of the overlay (B). Measurement at 214nm.

Figure 29 is an overlay of RP-HPLC chromatograms of the three different GM-CSF Control Samples (A), and an enlargement of the overlay (B). Measurement at 214 nm.

Figure 30 is an overlay of RP-HPLC chromatograms of GM-CSF drug substances from GM-CSF Control Sample 3 and from an existing commercial formulation (A), and an enlargement of the overlay (B). Measurement at 214nm.

Figure 31 is an overlay of RP-HPLC chromatograms of GM-CSF Control Sample 3 and drug product lyophilizates produced in Example 5 (A), and an enlargement of the overlay (B).

Figure 32 is an overlay of RP-HPLC chromatograms of lyophilized final formulation variant #1 produced in Example 5 to its corresponding placebo solution without drug substance GM-CSF (A), and an enlargement of the overlay (B). Measurement at 214 nm.

Figure 33 is an overlay of RP-HPLC chromatograms of lyophilized final formulation variant #2 produced in Example 5 to its corresponding placebo solution without drug substance GM-CSF (A), and an enlargement of the overlay (B). Measurement at 214 nm.

Figure 34 is an overlay of RP-HPLC chromatograms of GM-CSF Control Sample 3 and a positive control stressed at 60 °C for 18 hours (A), and an enlargement of the overlay (B).

Figure 35 is an overlay of SE-HPLC chromatograms of lyophilized final formulation variant #1 (TGWP7b/1-170814), lyophilized final formulation variant #2 (TGWP7b/2-170814), and lyophilized existing commercial formulation (A), and an enlargement of the overlay (B). Measurement at 214nm.

Figure 36 is an overlay of RP-HPLC chromatograms of lyophilized final formulation variant #1 (TGWP7b/1-170814), lyophilized final formulation variant #2 (TGWP7b/2-170814), and lyophilized existing commercial formulation (A), and an enlargement of the overlay (B). Measurement at 214nm.

Figure 37 is a photograph of lyophilized final formulation variants #1, #2, #1d and #2d directly after lyophilization (T=0).

Figure 38 is photographs of lyophilized final formulation variants #1, #2, #1d and #2d after T=8 weeks. A: stored at 25 °C; B: stored at 40 °C.

Figure 39 is a photograph of lyophilized final formulation variants #1, #2, #1d and #2d after T=12 weeks. Left: stored at 25°C; Right: stored at 40°C.

Figure 40 is a representative SE-HPLC chromatogram overlay recorded using 100 µL of 0.3mg/mL lyophilized final formulation variant #1 after storage at 40°C for 8 weeks, 12 weeks, and at T=0 (A), and an enlargement of the overlay (B). Measurement at 214nm. GM-CSF main peak at 33 minutes retention time, fragments detectable at 38 minutes retention time, aggregates at around 28 minutes.

Figure 41 is a graph showing the relative main peak areas of samples stored at 25 °C detected by SE-HPLC at 214nm.

Figure 42 is a graph showing the relative main peak areas of samples stored at 40°C detected by SE-HPLC at 214nm.

Figure 43 is a representative RP-HPLC chromatogram overlay recorded using 100 µL of 0.3 mg/mL GM-CSF lyophilizate variant #1 after storage at 40 C for 8 weeks, 12 weeks, and at T=0 (A), and an enlargement of the overlay (B). Measurement at 214nm. GM-CSF main peak at ~25 minutes retention time.

Figure 44 is a graph showing the relative main peak areas of samples stored at 25°C detected by RP-HPLC at 214nm.

Figure 45 is a graph showning the relative main peak areas of samples stored at 40°C detected by RP-HPLC at 214nm.

Figure 46 is a microscope photograph of inflammation at the injection site of the existing commercial formulation in animal no. 2. Overview, HE, lens x4.

Figure 47 is a microscope photograph of inflammation at the injection site of the existing commercial formulation in animal no. 2. Overview, HE, lens x40.

Figure 48 is a microscope photograph of inflammation at untreated control site 6 in animal no. 2. Overview, HE, lens x4.

Figure 49 is a microscope photograph of inflammation at untreated control site 6 in animal no. 2. Overview, HE, lens x40.

Figure 50 is a microscope photograph of inflammation at the final formulation variant #1 injection site in animal no. 6. Overview, HE, lens x4.

Figure 51 is a microscope photograph of inflammation at the final formulation variant #1 injection site in animal no. 6. Overview, HE, lens x40.

Figure 52 is a microscope photograph of inflammation at the untreated control site 6 in animal no. 6. Overview, HE, lens x4.

Figure 53 is a microscope photograph of inflammation at the untreated control site 6 in animal no. 6. Overview, HE, lens x40.

Figure 54 is a photograph of representative vials after the final formulation lyophilization feasibility test run, using sucrose and mannitol. Vials are shown from side view (upper row) and bottom view (lower row).

Figure 55 is an SE-HPLC chromatogram recorded using 100µL of 0.3mg/mL GM-CSF DS (Control Sample 3) diluted with running buffer (A), and an enlargement of the chromatogram (B). Measurement at 214nm. GM-CSF main peak at 33 minutes retention time.

Figure 56 is a SE-HPLC chromatogram overlay recorded using 100µL of 0.3mg/mL of each of final formulation variants #1-m, #2-m, #1d-m, #2d-m (A), and an enlargement of the overlay (B). Measurement at 214nm. GM-CSF main peak at 33 minutes retention time, fragments detectable at 38 minutes retention time, aggregates detectable at 28 minutes.

Figure 57 is a RP-HPLC chromatogram recorded using 100µL of 0.3mg/mL GM-CSF DS (Control Sample 3) diluted with dilution buffer (A), and an enlargement (B). Measurement at 214 nm. GM-CSF main peak at 25.5 minutes retention time.

Figure 58 is a RP-HPLC chromatogram overlay recorded using 100µL of 0.3mg/mL of each of final formulation variants #1-m, #2-m, #1d-m, #2d-m (A), and an enlargement (B). Measurement at 214nm. GM-CSF main peak at 26 minutes retention time.

Figure 59 is a graph showing the relative main peak area by RP-HPLC, detected at 214nm, of the Norm-Ject tuberculin syringe at 2-8°C.

Figure 60 is a graph showing the relative main peak area by RP-HPLC, detected at 214nm, of the Norm-Ject tuberculin syringe at 30°C.

Figure 61 is a graph showing the relative main peak area by RP-HPLC, detected at 214nm, of the Micro-Fine insulin syringe at 2-8°C.

Figure 62 is a graph showing the relative main peak area by RP-HPLC, detected at 214nm, of the Micro-Fine insulin syringe at 30°C.

Figure 63 is a graph showing GM-CSF concentrations after stress relative to T=0.

Figure 64 is a graph showing the relative main peak area of several final formulation variants, measured by SE-HPLC, after stress, relative to T=0.

Figure 65 is a graph showing the relative main peak area of several final formulation variants, measured by RP-HPLC, after stress, relative to T=0.

Brief Description of the Sequence Listing

SEQ ID NO: 1 shows the amino acid sequence of immature wild-type human GM-CSF.

SEQ ID NO: 2 shows the amino acid sequence of mature wild-type human GM-CSF.

SEQ ID NO: 3 shows the amino acid sequence of Molgramostim.

SEQ ID NO: 4 shows the amino acid sequence of Sargramostim.

SEQ ID NO: 5 shows the amino acid sequence of immature Human Serum Albumin (HSA).

Detailed Description

The present invention is based on a formulation of a GM-CSF protein which does not, or substantially does not, contain human serum albumin (HSA) or recombinant human albumin (rHA), but which has the same, or improved, properties as existing formulations of GM-CSF. The invention relates, in general terms, to a formulation of a GM-CSF protein and a surfactant, which contains no, or substantially no, HSA or rHA. The formulation of various embodiments of the invention has at least the same potency as previously known formulations of GM-CSF proteins, a higher purity than previously known formulations of GM-CSF proteins, and has good stability. The purity of the formulation of the invention can be measured more accurately, such that the purity and degradation products can be controlled more easily. This provides a much safer and higher quality formulation than previously available. Moreover, the absence of HSA and rHA overcomes problems with obtaining regulatory approval for these excipients. In some embodiments, the formulation of the present invention has an improved potency compared to previous formulations.

Throughout the description, except where otherwise stated, the units “mg/ml”, “w/v”, “mM” and “IU” are calculated when the formulation of the invention is a liquid or frozen liquid. Unless otherwise stated, the units “wt%” are calculated excluding water or WFI, except for residual water after lyophilisation.

GM-CSF

The GM-CSF protein used in preferred embodiments of the formulations of the present invention corresponds to mature, wild-type GM-CSF. In general, the GM-CSF protein has at least 60% sequence identity to the amino acid sequence set out in SEQ ID NO: 2. In some embodiments, the GM-CSF protein has at least 60%, 70%, 80%, 90%, 95%, 99% or 100% sequence identity to SEQ ID NO: 2. In some embodiments, the GM-CSF protein has an amino acid substitution at position 23 of SEQ ID NO: 2, to replace arginine with leucine. In such embodiments, the GM-CSF protein has at least 60%, 70%, 80%, 90%, 95%, 99% or 100% sequence identity to SEQ ID NO: 4. In other embodiments, the GM-CSF protein has the amino

acid sequence of mature wild-type GM-CSF, as described above, with an additional N-terminal methionine residue. In particularly preferred embodiments, the GM-CSF protein has at least 60%, 70%, 80%, 90%, 95%, 99% or 100% sequence identity to SEQ ID NO: 3, preferably 100% sequence identity to SEQ ID NO: 3. In such embodiments, the N-terminal methionine may be partially cleaved, meaning that the N-terminal methionine is not cleaved from every GM-CSF molecule.

The GM-CSF protein used in the formulations of the present invention may be non-glycosylated, partially glycosylated, fully glycosylated or hyper-glycosylated, as compared to the mature, wild-type GM-CSF. Thus, in some embodiments, no more than 25%, no more than 15%, no more than 12.5%, no more than 10%, no more than 5%, no more than 4%, no more than 3%, no more than 2%, or no more than 1% of the amino acid residues of the GM-CSF protein are glycosylated. In preferred embodiments, no more than 25% of the amino acid residues of the GM-CSF protein are glycosylated. In other preferred embodiments, no more than 12.5% of the amino acid residues of the GM-CSF protein are glycosylated. In yet further preferred embodiments, no more than 5% of the amino acid residues of the GM-CSF protein are glycosylated. In particularly preferred embodiments, none of the amino acid residues of the GM-CSF protein are glycosylated. In some embodiments, the percentage glycosylation discussed above is an average of the percentage glycosylation of the GM-CSF protein.

In some embodiments, the GM-CSF protein is glycosylated but has a different glycosylation pattern from mature, wild-type GM-CSF. For example, the GM-CSF protein may have a relative molecular weight of approximately 19,500, 16,800 or 15,500, or the GM-CSF protein may be a mixture of one or more of these three glycosylated forms of GM-CSF (i.e. a mixture of GM-CSF proteins having the above three molecular weights). For example, the GM-CSF protein may be a mixture of 25-42% of molecular weight 19,500 GM-CSF, 14-32% of molecular weight 16,800 GM-CSF and 35-53% of molecular weight 15,500 GM-CSF.

In preferred embodiments, the GM-CSF protein has at least 60%, 70%, 80%, 90%, 95%, 99% or 100% sequence identity to SEQ ID NO: 2, but is not glycosylated. In some embodiments, the GM-CSF protein has at least 60%, 70%, 80%, 90%, 95%, 99% or 100% sequence identity to the amino acid sequence of SEQ ID NO: 2, has an additional N-terminal methionine as compared to SEQ ID NO: 2, which may be partially cleaved, and is not glycosylated. In particularly preferred embodiments, GM-CSF used in the formulations of the present invention has at least 60%, 70%, 80%, 90%, 95%, 99% or 100% sequence identity to SEQ ID NO: 3, preferably 100% sequence identity to SEQ ID NO: 3, and is not glycosylated.

The GM-CSF protein used in the formulations of the present invention may comprise no more than 127 or 128 amino acid residues. In preferred embodiments, the GM-CSF protein has 128 amino acid residues. In some embodiments, the GM-CSF protein may be part of a fusion protein, wherein the fusion protein comprises more that 127 or 128 amino acid residues.

In embodiments, the GM-CSF protein used in the formulations of the present invention has cytokine activity, and is capable of activating local Dendritic Cells. The cytokine activity of GM-CSF can be measured by an *in vitro* bioassay, as described in "Monograph of Molgramostim Concentrated Solution" (European Pharmacopeia, 01/2008:1641). In particular, the biological activity of GM-CSF is determined by its stimulation of proliferation of TF-1 cells. The GM-CSF protein used in the formulations of embodiments of the present invention has at least 1% of the cytokine activity of mature, wild-type GM-CSF. In some embodiments, the GM-CSF protein has at least 10% of the cytokine activity of mature, wild-type GM-CSF.

The GM-CSF protein used in the formulations of the present invention may be produced by host cells including bacteria, yeast or Chinese Hamster Ovary (CHO) cells. In preferred embodiments, the GM-CSF protein is produced by *E. coli*. In other embodiments, the GM-CSF protein is produced by yeast, such as *Saccharomyces cerevisiae.*

The concentration of GM-CSF protein in the formulations of certain embodiments of the present invention may be adjusted to achieve a particular potency of the formulation for a given dose. In one embodiment, the concentration of the GM-CSF protein is such that a given dose of the formulation has a potency in the range of $0.26x10^6$ to $8.33x10^6$ IU. In some embodiments, the concentration of the GM-CSF protein is such that a given dose of the formulation has a potency in the range of $0.26x10^6$ to $0.42x10^6$ IU. In other embodiments, the concentration of the GM-CSF protein is such that a given dose of the formulation has a potency of $0.33x10^6$ IU. In some embodiments, a 100µl dose of the formulation has a potency in the range of $0.26x10^6$ to $0.42x10^6$ IU, preferably a potency of $0.33x10^6$ IU. In some embodiments, the concentration of GM-CSF is such that a given dose has a maximum potency of $3.3x10^6$ IU.

The GM-CSF protein may be present in the formulations at a concentration in the range of 0.001-10mg/ml, 0.01-10mg/ml, 0.05-10mg/ml, 0.1-10mg/ml, 0.05-5mg/ml, 0.05-2.5mg/ml, 0.05-1.5mg/ml, 0.1-1mg/ml, 0.1-0.5mg/ml or 0.1-0.4mg/ml. In some embodiments, the GM-CSF protein is present at a concentration of between 0.15 and 0.3mg/ml. In other embodiments, the GM-CSF protein is present in the formulations at a concentration of 0.15mg/ml or 0.3mg/ml. In

particularly preferred embodiments, the formulation of the present invention comprises 0.3mg/ml of the GM-CSF protein. In these embodiments, a 100µl dose of the formulation has a potency of $0.33x10^6$ IU.

HSA/rHA

The formulations of embodiments of the present invention include no, or substantially no, HSA or rHA. In particular, the formulations contain less than or equal to 0.2mg/ml, 0.15mg/ml, 0.1mg/ml, 0.05mg/ml, 0.02mg/ml or 0.01mg/ml HSA or rHA. Preferably, the formulations of the invention contain less than or equal to 0.02mg/ml HSA or rHA. In particularly preferred embodiments, the formulations contain no HSA or rHA (i.e. 0.0mg/ml HSA or rHA).

It has been found that HSA and rHA mask impurities in formulations of GM-CSF, as shown in Figure 7 and as discussed in Example 2. Thus, the presence of HSA or rHA makes it difficult to measure the purity of formulations of GM-CSF accurately. The formulations of embodiments of the present invention have the advantage that the purity thereof can be accurately measured because of the lack, or substantial lack, of HSA or rHA. Thus, the formulations of embodiments of the present invention are a higher quality product than previously-known formulations, because there is good control of the purity of the formulations.

In addition, the absence of HSA or rHA from the formulations of embodiments of the present invention means that the formulations avoid any regulatory difficulties that arise with the use of HSA and rHA.

Stabiliser

The formulations of the invention include a stabiliser which comprises a polymer or a surfactant. The polymer or surfactant may be selected from polymers and surfactants already known in the art, such as polyethylene glycol (PEG), gelatin, hydroxyethyl starch, dextran, dextran sulfate, polyethyleneimine (PEI), hydroxypropyl β-cyclodextrin, polyvinylpyrrolidone (PVP), hydroxyethylcellulose (HEC), poloxamers, Pluronic, N-octyl glucoside. In some instances, the polymer may act as a surfactant.

In some embodiments, the polymer is a poloxamer, and, in some embodiments, the polymer is Poloxamer 188 (P188). In other embodiments, the surfactant is Polysorbate 20 (P20) or Polysorbate 80 (P80). In preferred embodiments, the stabiliser comprises P188, as Example 2 and Figure 8 show that formulations containing P188 showed the best results in the forced degradation studies. In particular, Figure 8 shows that the monomer content of formulations with

P188 only drops below 99% for one variant, namely formulation #2 without air (sub-variant #2 (P188) N2). This high level of purity was also detected using RP-HPLC, as shown in Example 2 and Figure 12, with P188 providing the greatest stability.

In addition, it has been found that the use of P188 further increases the potency of the formulations of the invention, as shown in Table 85.

In some embodiments, the stabiliser comprising the polymer or the surfactant is present in an amount in the range of 0.005-10%w/v, 0.005-5%w/v, 0.01-10%w/v, 0.02-5%w/v, or 0.05-2%w/v, 0.05-1.5%w/v or 0.05-1%w/v. In some embodiments, the stabiliser comprising the polymer or the surfactant is present in an amount of 0.05%w/v. In other embodiments, the stabiliser comprising the polymer or the surfactant is present in an amount of 0.1%w/v. In some embodiments, the polymer or the surfactant is present in an amount in the range of 0.01-10%w/v, 0.02-5%w/v, or 0.05-2%w/v, 0.05-1.5%w/v or 0.05-1%w/v. In preferred embodiments, the polymer or surfactant is present in an amount of 0.05%w/v or 0.1%w/v. In particularly preferred embodiments, the polymer is P188 or the surfactant is P20 or P80, present in an amount of 0.05%w/v or 0.1%w/v.

In some embodiments, the stabiliser comprising the polymer or the surfactant is present in an amount of 0.1-5mg/ml, 0.2-2.5mg/ml, 0.2-1.5mg/ml, or 0.5-1.0mg/ml. In some embodiments, the stabiliser comprising the polymer or the surfactant is present in an amount of 0.5mg/ml. In other embodiments, the stabiliser comprising the polymer or the surfactant is present in an amount of 1.0mg/ml. In some embodiments, the polymer or the surfactant is present in an amount of 0.1-5mg/ml, 0.2-2.5mg/ml, 0.2-1.5mg/ml, or 0.5-1.0mg/ml. In preferred embodiments, the polymer or the surfactant is present in an amount of 0.5mg/ml or 1.0mg/ml. In particularly preferred embodiments, the polymer is P188 or the surfactant is P20 or P80, present in an amount of 0.5mg/ml or 1.0mg/ml.

The stabiliser may comprise one or more further components, for example, further components known in the art to act as stabilisers. In some embodiments, the stabiliser may further comprise a disaccharide or an amino acid. In some embodiments, the disaccharide is sucrose, trehalose, lactose, inositol and/or mannitol, and the amino acid is arginine and/or glycine. In some embodiments, the disaccharide is mannitol. In some embodiments, the amino acid is arginine. In preferred embodiments, the disaccharide is sucrose. In other embodiments, the stabilizer further comprises sucrose and a second further component, which may be a disaccharide and/or an amino acid as defined above. In some embodiments, the further components are

sucrose and mannitol, sucrose and glycine, sucrose and trehalose, or sucrose and arginine. In particular, the use of mannitol, glycine, trehalose or arginine in combination with sucrose may improve the lyo cake resulting from lyophilisation, by reducing the amount of shrinkage of the lyo cake.

Advantageously, it was found that the use of a disaccharide may improve the potency of the formulation, as shown in Table 85.

It was also found, as shown in Table 4, that formulations containing sucrose have a higher thermodynamic stability, higher colloidal stability and a higher unfolding enthalpy than other formulations tested, showing that formulations containing sucrose are more stable than other formulations tested. Example 2 also shows that formulations containing sucrose show the least chemical degradation and have the lowest aggregate status.

It has also been found that when the stabiliser further comprises arginine, the formulation had a high T_{onset} and A_2, as shown in Table 4, indicating a good ability to stabilise the GM-CSF protein. In addition, Example 2 shows that formulations containing arginine have good results with regard to chemical degradation and aggregate status.

In other embodiments, the disaccharide is a mixture of sucrose and mannitol. In such embodiments, the presence of mannitol reduces the amount of shrinkage of the lyo cake, following lyophilisation of the formulation, as discussed in Example 12. An improved lyo cake has the advantage that the correct dosage is more reliably reconstituted because, for example, a more solid lyo cake has a reduced risk of fragmentation, wherein fragmentation can affect the dose in vials with a low fill volume. In particular, a more solid lyo cake has a reduced risk of fragments or powder from the cake sticking to the vial stopper. Example 14 shows that such formulations are suitable for use as a lyophilized drug product.

The one or more further components may be present at a concentration in the range of 5-1000mM, 5-500mM, 50-500mM, 100-500mM, 100-400mM or 200-300mM. In all embodiments, the formulation is isotonic to the physiological conditions.

In some embodiments, sucrose is present in the formulation at a concentration of 5-1000mM, 5-500mM, 10-400mM, 50-400nM, 100-400mM, 100-300mM or 200-300mM. In preferred embodiments, sucrose is present in the formulation at a concentration of 260mM. In other embodiments, sucrose is present in the formulation at a concentration of 130mM.

Mannitol may be present in the formulation at a concentration of 5-1000mM, 5-500mM, 50-500mM, 100-500mM, 100-400mM, 100-300mM or 100-250mM. In some embodiments, mannitol is present in the formulation at a concentration of 220mM. In other embodiments, mannitol is present at a concentration of 110mM.

Arginine may be present in the formulation at a concentration of 5-1000mM, 5-500mM, 20-400mM, 100-400mM, 100-300mM or 100-250mM. In some embodiments, arginine is present in the formulation at a concentration of 260mM. In other embodiments, arginine is present at a concentration of 130mM.

Glycine may be present in the formulation at a concentration of 5-1000mM, 5-500mM, 50-500mM, 100-500mM, 100-400mM, 100-300mM or 100-250mM. In some embodiments, glycine is present in the formulation at a concentration of 220mM. In other embodiments, glycine is present at a concentration of 110mM.

When the stabiliser contains more than one further component, the total concentration of the further components does not exceed 500mM in some embodiments, and the formulation is isotonic to the physiological conditions.

In embodiments wherein the stabilizer further comprises sucrose and a second further component, the ratio of sucrose to the second further component may be 1:2. In some embodiments, sucrose is present in the formulation at a concentration of 5-500mM, and the second further component is present in the formulation at a concentration of 5-500mM. In some embodiments, sucrose is present in the formulation at a concentration of 10-300mM, 10-250mM, 10-200, 10-150 or 10-100mM, and the second further component is present at a concentration of 50-300mM, 50-250mM or 100-250mM. In some embodiments, sucrose is present in the formulation at a concentration of 58mM and the second further component is present at a concentration of 220mM. In other embodiments, sucrose is present in the formulation at a concentration of 29mM and the second further component is present at a concentration of 110mM.

Buffer

The formulations of embodiments of the present invention may comprise a buffer, such as potassium phosphate, disodium phosphate, potassium dihydrogen phosphate or Tris. The

buffer maintains the pH of the formulations of the invention in the range of 6.5 to 9.5, 7.0 to 8.5 or, more preferably, in the range of 7.3 to 8.1.

In preferred embodiments, the buffer is potassium phosphate. In particular, potassium phosphate also acts as a stabilizer, and Table 2 shows that potassium phosphate was found to be good at stabilising the GM-CSF protein, with weaker aggregation (i.e. a less negative A_2) than other buffers. Table 2 also shows that formulations comprising potassium phosphate as the buffer had a high T_{onset}, T_M and delta H.

In addition, if the GM-CSF drug substance (i.e. unformulated) has been synthesised using potassium phosphate as a buffer, then it is advantageous also to use potassium phosphate as the buffer for the formulated drug product as it avoids the need for an additional processing step during preparation of the formulation.

In other embodiments, the buffer may be Tris. In particular, Table 2 shows that Tris provides weak aggregation (i.e. a positive A_2), and that formulations comprising Tri had a high T_{onset}, T_M and delta H.

In some embodiments, the concentration of the buffer is at least 0.1mM, at least 1mM, at least 2mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 11mM, at least 12mM, at least 13mM, at least 14mM or is at least 15mM. In some embodiments, the concentration of the buffer is no more than 40mM, 30mM, 20mM or 10mM.

Preferably, the buffer is present at a concentration between 1mM and 40mM, between 5mM and 40mM or between 5mM and 15mM. More preferably, the concentration of the buffer is 5mM or 10mM. In a preferred embodiment, the buffer is potassium phosphate at a concentration of 5mM or 10mM.

In some embodiments, the concentration of the buffer is at least 0.1mg/ml, at least 0.2mg/ml, at least 0.3mg/ml, at least 0.4mg/ml, at least 0.5mg/ml, at least 0.6mg/ml, at least 0.7mg/ml, at least 0.80mg/ml, at least 0.85mg/ml, at least 0.9mg/ml, at least 0.95mg/ml, at least 0.96mg/ml, at least 0.97mg/ml, at least 0.98mg/ml, at least 0.99mg/ml or is at least 1.0mg/ml. In preferred embodiments, the concentration of buffer is 0.98mg/ml.

pH

The formulation of the present invention may have a pH of at least 6.5, at least 6.6, at least 6.7, at least 6.8, at least 6.9, at least 7.0, at least 7.1, at least 7.2, at least 7.3, at least 7.4, at least 7.5, at least 7.6, at least 7.7, at least 7.8, at least 7.9, at least 8.0, at least 8.1, at least 8.2, at least 8.3, at least 8.4, at least 8.5, at least 8.6, at least 8.7, at least 8.8, at least 8.8 or at least 9.0, and a maximum pH of 9.5, 9.4, 9.3, 9.2, 9.1, 9.0, 8.9, 8.8, 8.8, 8.6 or 8.5. Preferably, the formulation has a pH in the range of 6.5 to 9.5, 7.0 to 8.5, and more preferably in the range of 7.3 to 8.2. In particularly preferred embodiments, the formulation has a pH of 7.3, 8.1 or 8.2, most preferably 7.3 or 8.1. In some embodiments, the pH is measured after reconstitution of the formulation in WFI.

From the pre-screening in Example 1 (Table 2), it was determined that a more basic environment had a more beneficial effect on colloidal stability, while the first and second DoE matrices of Example 1 showed that formulations having a pH of 7.3 or 8.1 have good colloidal and thermodynamic stability. Example 3 also shows this particularly good colloidal stability and thermodynamic property of formulations having a pH of 7.3 or 8.1, which have excellent results in the nanoparticle analysis.

In some embodiments, it is preferred that the formulation has pH 8.1. In particular, Figures 3 and 4 show that the A_2 value increases (indicating less aggregation) with increasing pH and that T_{onset} increase with increasing buffer molality. In addition, Tables 4 and 28 show that a pH of 8.1 provides for less aggregation, and Figures 8 and 12 show that formulations having pH 8.1 (i.e. sub-variants derived from test variants #3 and #4) have the best purity.

In addition, it has been found that embodiments of formulations of the present invention having a pH in the range of 7.0-8.5 have good purity. Table 24 shows that more than 99% of nanoparticles in the formulation had a radius in the size range of 0.1-10nm, even when temperature-stressed. Examples 10 and 11 also show that such formulations have good potency, which at least meets the acceptance criterion for minimal potency of human GM-CSF (Tables 85 and 95).Moreover, Example 9 shows that formulations of the invention, and particularly formulations having pH8.1, are less of an irritant than previously known formulations of GM-CSF. For example, Example 9 shows that, at day 24 after injection of the formulation, formulations of the present invention having pH8.1 were less irritating than an existing commercial formulation and formulations having pH7.3.

Thus, in embodiments comprising sucrose, mannitol, or sucrose and mannitol, the pH of the formulation is preferably 7.3 or 8.1, preferably 8.1. As mentioned above, formulations having these pHs have good colloidal and thermodynamic stability, low aggregation and good purity, and formulations having pH 8.1 show the best properties.

In embodiments comprising arginine, the pH of the formulation is preferably 8.1. In particular, as shown in Example 2, section 4.3.2, and Figure 12, formulations comprising arginine and having a pH of 8.1 show a good purity, with a relative main peak area of between 70% and 90%.

The formulations of the present invention can be adjusted during preparation to the requisite pH as required, for example using NaOH, KOH or HCl.

wt% of each component

The formulations of the present invention may comprise each component within a range of weight percent as will now be described (except where otherwise stated, weight percent is calculated below excluding WFI except residual water after lyophilization). In some embodiments, GM-CSF is present in the range of 0.001-10wt%, 0.01-10wt%, 0.05-10wt%, 0.1-10wt%, 0.05-5wt%, 0.05-2.5wt%, 0.05-1.5wt%, 0.1-1wt%, 0.1-0.7wt% or 0.3-0.7wt%. In some embodiments, GM-CSF is present in the range of 0.3-0.5wt%. In some embodiments, GM-CSF is present at 0.33wt%, 0.48wt%, 0.60wt% or 0.63wt%.

In some embodiments, the stabiliser is present in the range of 0.005-99.0wt%, 0.01-99.0wt%, 0.05-99.0wt%, 0.1-99.0wt%, 0.5-99.0wt%, 1.0-99.0wt%, 1.0-98.0wt%, 1.0-97.5wt%, 1.1-98wt% or 1.1-97.5wt%. The polymer or surfactant is present in the range of 0.005-10.0wt%, 0.01-10.0wt%, 0.05-10.0wt%, 0.05-5.0wt%, 0.5-5.0wt%, 0.5-3.0wt%, or 1.0-3.0wt%. In some embodiments, the polymer or surfactant is present in the range of 1.0-2.5wt%, and preferably in the range of 1.0-2.0wt%. In some embodiments, the polymer or surfactant is present at 1.1wt%, 1.6wt%, 2.0wt% or 2.1wt%.

When the stabiliser comprises one or more further components, the one or more further components, such as a disaccharide or arginine, may be present in total in the range of 10.0-99.0wt%, 20.0-99.0wt%, 30.0-99.0wt%, 40.0-99.0wt%, 50.0-99.0wt%, 60.0-99.0wt%, 70.0-99.0wt%, 80.0-99.0wt%, 90.0-99.0wt%, 90.0-98.0wt%, 92.0-98.0wt% or 95.0-98.0wt%. In some embodiments, the one or more further components is present in the range of 96.0-98.0wt%.

When the stabiliser comprises sucrose, sucrose may be present in the range of 10.0-99.0wt%, 20.0-99.0wt%, 30.0-99.0wt%, 40.0-99.0wt%, 50.0-99.0wt%, 60.0-99.0wt%, 70.0-99.0wt%, 80.0-99.0wt%, 90.0-99.0wt%, 90.0-98.0wt%, 92.0-98.0wt% or 95.0-98.0wt%. In some embodiments, sucrose is present in the range of 97.0-98.0wt%. In some embodiments, sucrose is present at 97.5wt%.

When the stabiliser comprises mannitol, mannitol may be present in the range of 10.0-99.0wt%, 20.0-99.0wt%, 30.0-99.0wt%, 40.0-99.0wt%, 50.0-99.0wt%, 60.0-99.0wt%, 70.0-99.0wt%, 80.0-99.0wt%, 90.0-99.0wt%, 90.0-98.0wt%, 92.0-98.0wt% or 95.0-98.0wt%. In some embodiments, mannitol is present in the range of 95.0-96.0wt%. In some embodiments, mannitol is present at 95.4wt%.

When the stabiliser comprises sucrose and mannitol, each of sucrose and mannitol may be present in the range of 10.0-99.0wt%, 20.0-99.0wt%, 30.0-99.0wt%, 40.0-99.0wt%, 50.0-99.0wt%, 60.0-99.0wt%, 70.0-99.0wt%, 80.0-99.0wt%, 90.0-99.0wt%, 90.0-98.0wt%, 92.0-98.0wt% or 95.0-98.0wt%. In some embodiments, sucrose is present in the range of 15.0-50.0wt%, 25.0-35.0wt%, 27.0-33.0wt% or 28.0-32.0wt%, and mannitol is present in the range of 45.0-85.0wt%, 60.0-70.0wt%, 60.0-67.0wt%, or 62.0-67.0wt%. In some embodiments, sucrose is present in the range of 30.0-32.0wt%, and mannitol is present in the range of 64.0-65.0wt%. In some embodiments, sucrose is present at 31.7wt% and mannitol is present at 64.8wt%.

When the stabiliser comprises arginine, arginine may be present in the range of 10.0-99.0wt%, 20.0-99.0wt%, 30.0-99.0wt%, 40.0-99.0wt%, 50.0-99.0wt%, 60.0-99.0wt%, 70.0-99.0wt%, 80.0-99.0wt%, 90.0-99.0wt%, 90.0-98.0wt%, 92.0-98.0wt%, 93.0-98.0wt%, 94.0-98.0wt% or 95.0-98.0wt%. In some embodiments, arginine is present in the range of 95.0-97.0wt% or 95.0-96.0wt%. In some embodiments, arginine is present at 95.3wt%.

In some embodiments, the buffer is present in the range of 0.1-10.0wt%, 0.1-5.0wt%, 0.1-3.0wt%, 0.5-5.0wt%, 0.5-2.5wt%, 1.0-2.5wt% or 1.0-2.0wt%. In some embodiments, the buffer is present in the range of 1.0-2.0wt%. In some embodiments, the buffer is present at 1.0wt%, 1.5wt%, 1.9wt% or 2.0wt%.

The formulations of embodiments of the present invention contain HSA or rHA in an amount less than or equal to 0.20wt%, 0.15wt%, 0.10wt%, 0.050wt%, 0.030wt%, 0.020wt%, 0.010wt%, 0.005wt%, 0.003wt%, 0.002wt% or 0.001wt%. In preferred embodiments, HSA or rHA is

present in the formulations of the invention in an amount less than or equal to 0.02wt%. In some embodiments, the formulations of the invention contain no (i.e. 0.0wt%) HSA or rHA.

In embodiments where the formulation comprises water, water may be present at at least 10.0wt%, 20.0wt%, 30.0wt%, 40.0wt%, 50.0wt%, 60.0wt%, 65.0wt%, 70.0wt%, 75.0wt%, 80.0wt%, 85.0wt%, 90.0wt%, 91.0wt%, 92.0wt%, 93.0wt%, 94.0wt%, 95.0wt%, 95.5wt%, 96.0wt%, 96.5wt%, 97.0wt%, 97.5wt%, 98.0wt%, 98.5wt% or 99.0wt%.

In embodiments where the formulation comprises water, water may be present in the range of 10.0-99.0wt%, 20.0-99.0wt%, 30.0-99.0wt%, 40.0-99.0wt%, 50.0-99.0wt%, 60.0-99.0wt%, 70.0-99.0wt%, 80.0-99.0wt%, 85.0-99.0wt%, 90.0-99.0wt%, 91.0-99.0wt%, 91.0-98.5wt% or 91.0-98.0wt%. In some embodiments, water is present in the range of 85.0-97.5wt%, 90.0-97.5wt%, 91.0-97.5wt%, 91.0-96.0wt%, 91.0-95.5wt% or 91.5-95.5wt%. In other embodiments, water is present in the range of 85.0-99.0wt%, 90.0-99.0wt%, 93.0-99.0wt%, 95.0-99.0wt%, 95.0-98.0wt% or 95.0-98.0wt%. In some embodiments, water is present at 91.6wt%, 94.1wt%, 95.2wt% or 95.4wt%. In other embodiments, water is present at 95.6wt%, 97.0wt%, 97.6wt% or 97.7wt%.

In one embodiment, the formulation comprises GM-CSF in the range of 0.1-2.5wt%, P188 in the range of 0.5-5.0wt%, sucrose in the range of 70.0-99.0wt% and potassium phosphate in the range of 0.1-2.5wt%. In some embodiments, the formulation comprises GM-CSF in the range of 0.30-0.36wt%, P188 in the range of 1.0-1.2wt%, sucrose in the range of 87.5-97.8wt% and potassium phosphate in the range of 0.9-1.2wt%. When water is present, it may be in the range of 82.4-99.0wt% or in the range of 86.0-99.0wt%.

In one embodiment, the formulation comprises GM-CSF in the range of 0.1-2.5wt%, P188 in the range of 0.5-5.0wt%, mannitol in the range of 70.0-99.0wt% and potassium phosphate in the range of 0.1-3.0wt%. In some embodiments, the formulation comprises GM-CSF in the range of 0.5-0.7wt%, P188 in the range of 1.9-2.1wt%, mannitol in the range of 85.9-95.9wt% and potassium phosphate in the range of 1.7-2.1wt%. When water is present, it may be in the range of 85.7-99.0wt% or in the range of 87.8-99.9wt%.

In one embodiment, the formulation comprises GM-CSF in the range of 0.1-2.5wt%, P188 in the range of 0.5-5.0wt%, sucrose in the range of 15.0-50.0wt%, mannitol is present in the range of 45.0-85wt% and potassium phosphate is present in the range of 0.1-3.0wt%. In some embodiments, the formulation comprises GM-CSF in the range of 0.4-0.5wt%, P188 in the

range of 1.4-1.8wt%, sucrose in the range of 28.6-35.0wt%, mannitol in the range of 58.1-71.0wt% and potassium phosphate in the range of 1.4-1.7wt%. When water is present, it may be in the range of 84.7-99.0wt% or in the range of 87.3-99.0wt%.

In one embodiment, the formulation comprises GM-CSf in the range of 0.1-2.5wt%, P188 in the range of 0.5-5.0wt%, arginine in the range of 70.0-99.0wt% and potassium phosphate in the range of 0.1-3.0wt%. In some embodiments, the formulation comprises GM-CSF in the range of 0.6-0.7wt%, P188 in the range of 1.9-2.3wt%, arginine in the range of 85.7-95.7wt% and potassium phosphate in the range of 1.8-2.2wt%. When water is present, it may be present in the range of 85.9-99.0wt% or in the range of 87.9-99.0wt%.

Purity

The embodiments of the formulations of the present invention have good purity, as shown in Examples 2 and 3. In contrast, the recorded purity of the previously-known formulations should be considered to be inaccurate and difficult to measure, because it has been found that HSA and rHA have a masking effect on purity measurements. Example 2 (Figure 7) and Example 7 (Figures 35 and 36) show that HSA masks the potential aggregates of GM-CSF, and that the results from samples containing rHA cannot be considered exact as the main aggregation peaks from rHA and the main aggregation peaks from GM-CSF were not separated. In addition, Figure 11 shows that rHA masks the impurities in formulations containing rHA, such that these formulations appear to be purer than they actually are (see Example 2).

The purity of the formulation was also tested after reconstitution from lyophilisates, and was shown to be very good for formulations of the present invention. In particular, Table 40 shows that formulations of embodiments of the present invention had an aggregate content of less than 2%, while Table 41 shows that formulations of embodiments of the present invention have a purity of at least 97%. Moreover, Table 56 shows that formulations of the invention had an aggregate content of less than 2%, while Table 57 shows that formulations of the invention had a purity of over 96%, even after having been held at 40°C for 12 weeks. In contrast, it was not possible to measure the purity of reconstituted lyophilisates of existing commercial formulations, as potential aggregate and impurity peaks were masked by the HSA peak.

In some embodiments, the formulation of embodiments of the present invention has a purity of at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100%, when measured after 8, or 12 weeks of storage.

Aggregation status/nano-particles

The formulations of the present invention have the vast majority of their nanoparticles in the smallest size range measured (i.e. 0.1-10nm), even after reconstitution from a lyophilisate, thereby indicating that the risk of aggregation of the formulations of the present invention is low. In particular, Table 42 shows that, in nearly all reconstituted formulations of the present invention, more than 98% of the nanoparticles had a radius in the range of 0.1-10nm, and had an average radius of about 2.0nm. In contrast, the reconstituted existing commercial formulation (#C) showed only 95% of nanoparticles being in the range of 0.1-10nm, with average radius which was twice that of the formulations of the present invention, namely, 4.3nm. In addition, 4.8% of the nanoparticles in the reconstituted existing commercial formulation (#C) had a radius in the range of 100-1000nm. Furthermore, even after stress, the formulations of the present invention continue to have the vast majority of nanoparticles in the size range of 0.1-10nm (Example 8, Table 58). In particular, Table 58 shows that, even after storage at 25°C and 40°C for 8 or 12 weeks, the formulations had a mean mass fraction in the size range of 0.1-10nm of more than 93%. Thus, the formulations of the present invention have a very good purity, and this indicates that the European Pharmacopoeia requirement for the number of sub-visible particles (maximum 6000 particles of 10µm and maximum 600 particles of 25µm, in a volume of 25ml or at least 25ml) is expected to be met, even when the process of producing the formulations of the present invention is scaled up.

In some embodiments, the formulations of the present invention have an aggregate content of nanoparticles having a radius greater than 10nm of less than 4.5%, less than 3.5%, less than 2.5% or, preferably, less than 2%. In some embodiments, the formulations have an average nanoparticle radius in the range of 0.1-10nm, 0.5-10nm, 0.5-5nm, 1-5nm, or, preferably, 1-3nm. In some embodiments, the average nanoparticle diameter is about 2nm.

Residual water

The formulation of embodiments of the present invention also provides the advantage that, after lyophilisation, there is a reduced amount of residual water compared to previously known formulations, thereby reducing the risk of spoilage of the formulation. As shown in Table 38, the residual water in formulations of the present invention was well below 1.0%, with the maximum residual water being 0.5%, while the control formulation (an existing commercial formulation) had 0.6% residual water. Moreover, Table 54 shows that the residual water in the lyophilised product remained well below 1% even after forced degradation using temperature.

In some embodiments, the formulation has a maximum residual water content, immediately after lyophilisation, of 1.0% or 0.5%. In some embodiments, the formulation has a maximum residual water content, after storage at 25°C for 12 weeks, of 1% or 0.6%.

Moreover, lyophilizates of the formulations of the present invention can be reconstituted in WFI in less than 10 seconds, which is more rapid than existing commercial formulations of GM-CSF (Example 8).

Fill volumes of vials

The formulation of the present invention is stored in vials, at a volume in the range of 0.1-2.0ml. In some embodiments, the volume of formulation per vial is in the range of 0.1-1.0ml, 0.2-0.9ml, or 0.3-0.8ml. In some embodiments, the vials are filled to a volume of 0.1ml. In other embodiments, the volume of formulation per vial is 0.33ml. In some embodiments, the volume of formulation per vial is 0.66ml. In further embodiments, the volume of formulation per vial is 1ml. In embodiments where the volume of formulation per vial is 0.66ml, each vial contains the same amount of each ingredient, except that each vial contains twice the amount of water for injection (WFI), such that the formulations in the vials filled with 0.66ml of formulation are twice as dilute compared to the formulation in the vials filled with 0.33ml of formulation. For example, in embodiments where the formulation comprises 0.3mg/ml GM-CSF protein, vials containing 0.33ml of the formulation will contain 0.1mg of GM-CSF protein. Vials containing 0.66ml of this same formulation will also contain 0.1mg of GM-CSF protein, but will contain twice as much WFI as corresponding vials having a fill volume of 0.33ml.

It has been found that a fill volume of 0.66ml, which is twice the fill volume of previous formulations of GM-CSF, provides the advantage that there is less aggregation (Example 4; Table 28), and that it is easier to fill the vials accurately.

In some embodiments, each vial contains a maximum dose of GM-CSF of 2.0mg, 1.0mg, 0.5mg, 0.4mg, 0.3mg, 0.2mg, 0.1mg or 0.05mg. Preferably, each vial contains no more than 1.0mg of GM-CSF.

In some instance, the vial is a syringe, and the dose or fill volume is the same as described above. This provides the advantage that the risk of transferring an incomplete dose from vial to syringe is avoided.

Specific formulations

In some embodiments of the formulation of the present invention, the formulation consists of a GM-CSF protein having at least 60% sequence identity, and preferably 100% sequence identity, to SEQ ID NO: 3, potassium phosphate, sucrose and P188. The formulation is made up to a final volume with WFI. The liquid formulation has a pH of 7.3 or 8.1, preferably 8.1.

In other embodiments of the formulation of the present invention, the formulation consists of a GM-CSF protein having at least 60% sequence identity, and preferably 100% sequence identity, to SEQ ID NO: 3, potassium phosphate, arginine/ H_3PO_4 and P188. The formulation is made us to a final volume with WFI. The liquid formulation has a pH of 7.3 or 8.1, preferably 8.1.

In some embodiments of the formulation of the present invention, the formulation consists of a GM-CSF protein having at least 60% sequence identity, and preferably 100% sequence identity, to SEQ ID NO: 3, potassium phosphate, mannitol and P188. The formulation is made up to a final volume with WFI. The liquid formulation has a pH of 7.3 or 8.1, preferably 8.1.

In further embodiments of the formulation of the present invention, the formulation consists of a GM-CSF protein having at least 60% sequence identity, and preferably 100% sequence identity, to SEQ ID NO: 3, potassium phosphate, sucrose, mannitol and P188. The formulation is made us to a final volume with WFI. The liquid formulation has a pH of 7.3 or 8.1, preferably 8.1.

In a specific embodiment of the formulations of the present invention, the formulation consists of 0.3mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 10mM potassium phosphate, 260mM sucrose, 0.1% w/v P188, and has a pH of 7.3. A liquid formulation is made up to 0.33ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 29.4mg sucrose and 0.33mg P188, and the liquid formulation is made up to 0.33ml with WFI. The pH is adjusted to 7.3 using potassium hydroxide. This formulation, in particular, has been found to have a good purity, which can be accurately measured, and a good stability (Figures 41, 42, 44 and 45). This formulation was found to have some of the best overall results (Example 2), and Examples 10 and 11 (Tables 85 and 95) show that this formulation has at least the same potency as previously used formulations, and meets the criterion for minimal potency of human GM-CSF.

In another embodiment of the formulations of the present invention, the formulation consists of 0.15mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 5mM potassium phosphate, 130mM sucrose, 0.05% w/v P188, and has a pH of 7.3. The liquid formulation is

made up to 0.66ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 29.4mg sucrose and 0.33mg P188, and the liquid formulation is made up to 0.66ml with WFI. The pH is adjusted to 7.3 using potassium hydroxide. As discussed above, it was found that the increased dilution of the formulation decreased the amount of aggregation in the formulation (Example 4; Table 28) and that vials could be more accurately filled when using a greater fill volume.

In an alternative embodiment of the formulations of the present invention, the formulation consists of 0.3mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 10mM potassium phosphate, 260mM sucrose, 0.1% w/v P188, and has a pH of 8.1. The liquid formulation is made up to 0.33ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 29.4mg sucrose and 0.33mg P188, and the liquid formulation is made up to 0.33ml with WFI. The pH is adjusted to 8.1 using potassium hydroxide. This formulation, in particular, has been found to have a good purity, which can be accurately measured, and a good stability (Figures 41, 42, 44 and 45). This formulation was found to have some of the best overall results (Example 2), and is the least irritating (Example 9). Moreover, Examples 10 and 11 (Tables 85 and 95) show that this formulation has at least the same potency as previously used formulations, and meets the criterion for minimal potency of human GM-CSF.

In a further embodiment, the formulation consists of 0.15mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 5mM potassium phosphate, 130mM sucrose, 0.05% w/v P188, and has a pH of 8.1. The liquid formulation is made up to 0.66ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 29.4mg sucrose and 0.33mg P188, and the liquid formulation is made up to 0.66ml with WFI. The pH is adjusted to 8.1 using potassium hydroxide. As discussed above, it was found that the increased dilution of the formulation decreased the amount of aggregation in the formulation (Example 4; Table 28) and that vials could be more accurately filled when using a greater fill volume.

In another embodiment, the formulation consists of 0.3mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 10mM potassium phosphate, 260mM Arginine/H_3PO_4, 0.1%w/v P188, and has a pH of 7.3. The liquid formulation is made up to 0.33ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 15.1mg Arginine/H_3PO_4 and 0.33mg P188, and the liquid formulation is made up to 0.33ml with WFI. The pH is adjusted to 7.3 using potassium hydroxide.

In an alternative embodiment, the formulation consists of 0.15mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 5mM potassium phosphate, 130mM Arginine/H_3PO_4, 0.05% w/v P188, and has a pH of 7.3. The liquid formulation is made up to 0.66ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 15.1mg Arginine/H_3PO_4 and 0.33mg P188, and the liquid formulation is made up to 0.66ml with WFI. The pH is adjusted to 7.3 using potassium hydroxide.

In one embodiment, the formulation consists of 0.3mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 10mM potassium phosphate, 260mM Arginine/H_3PO_4, 0.1% w/v P188 and has a pH of 8.1. The liquid formulation is made up to 0.33ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 15.1mg Arginine/H_3PO_4 and 0.33mg P188, and the liquid formulation is made up to 0.33ml with WFI. The pH is adjusted to 8.1 using potassium hydroxide.

In another embodiment, the formulation consists of 0.15mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 5mM potassium phosphate, 130mM Arginine/H_3PO_4, 0.05% w/v P188, and has a pH of 8.1. The liquid formulation is made up to 0.66ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 15.1mg Arginine/H_3PO_4 and 0.33mg P188, and the liquid formulation is made up to 0.66ml with WFI. The pH is adjusted to 8.1 using potassium hydroxide.

In one embodiment, the formulation consists of 0.3mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 10mM potassium phosphate, 260mM mannitol, 0.1%w/v P188 and has a pH of 7.3. The liquid formulation is made up to 0.33ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 15.6mg mannitol and 0.33mg P188, and the liquid formulation is made up to 0.33ml with WFI. The pH is adjusted to 7.3 using potassium hydroxide.

In another embodiment, the formulation consists of 0.15mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 5mM potassium phosphate, 130mM mannitol, 0.05% w/v P188 and has a pH of 7.3. The liquid formulation is made up to 0.66ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 15.6mg mannitol and 0.33mg P188, and the liquid formulation is made up to 0.66ml with WFI. The pH is adjusted to 7.3 using potassium hydroxide.

In an alternative embodiment, the formulation consists of 0.3mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 10mM potassium phosphate, 260mM mannitol, 0.1% w/v P188, and has a pH of 8.1. The liquid formulation is made up to 0.33ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 15.6mg mannitol and 0.33mg P188, and the liquid formulation is made up to 0.33ml with WFI. The pH is adjusted to 8.1 using potassium hydroxide.

In a further embodiment, the formulation consists of 0.15mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 5mM potassium phosphate, 130mM mannitol, 0.05% w/v P188, and has a pH of 8.1. The liquid formulation is made up to 0.66ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 15.6mg mannitol and 0.33mg P188, and the liquid formulation is made up to 0.66ml with WFI. The pH is adjusted to 8.1 using potassium hydroxide.

In one embodiment, the formulation consists of 0.3mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 10mM potassium phosphate, 220mM mannitol, 58mM sucrose, 0.1% w/v P188, and has a pH of 7.3. The liquid formulation is made up to 0.33ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 13.5mg mannitol, 6.6mg sucrose and 0.33mg P188, and the liquid formulation is made up to 0.33ml with WFI. The pH is adjusted to 7.3 using potassium hydroxide.

In another embodiment, the formulation consists of 0.15mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 5mM potassium phosphate, 110mM mannitol, 29mM sucrose, 0.05% w/v P188, and has a pH of 7.3. The liquid formulation is made up to 0.66ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 13.5mg mannitol, 6.6mg sucrose and 0.33mg P188, and the liquid formulation is made up to 0.66ml with WFI. The pH is adjusted to 7.3 using potassium hydroxide.

In one embodiment, the formulation consists of 0.3mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 10mM potassium phosphate, 220mM mannitol, 58mM sucrose, 0.1% w/v P188, and has a pH of 8.1. The liquid formulation is made up to 0.33ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 13.5mg mannitol, 6.6mg sucrose and 0.33mg P188, and the

liquid formulation is made up to 0.33ml with WFI. The pH is adjusted to 8.1 using potassium hydroxide.

In another embodiment, the formulation consists of 0.15mg/ml GM-CSF protein having 100% sequence identity to SEQ ID NO: 3, 5mM potassium phosphate, 110mM mannitol, 29mM sucrose, 0.05% w/v P188, and has a pH of 8.1. The liquid formulation is made up to 0.66ml with WFI. More specifically, in this embodiment, the formulation consists of 0.1mg GM-CSF protein, 0.32mg potassium phosphate, 13.5mg mannitol, 6.6mg sucrose and 0.33mg P188, and the liquid formulation is made up to 0.66ml with WFI. The pH is adjusted to 8.1 using potassium hydroxide.

The formulation of the present invention can be in one of several physical states. For example, the formulation may be a liquid, a frozen liquid, or a freeze-dried lyophilizate. In embodiments where the formulation a liquid, the components of the formulation are as described above, with the addition of WFI to the required volume. The liquid formulation may be prepared by adding WFI to the components of the formulation, or by adding water to a lyophilised formulation.

In embodiments where the formulation is a frozen liquid, the formulation comprises the components are as described above, with the addition of WFI, in frozen form.

In embodiments where the formulation is a freeze-dried lyophilizate, the lyophilizate preferably contains less than 5% residual water, and preferably less than 1% residual water. The lyophilized formulation comprises the components aside from water as described above, and can be reconstituted for use by the addition of WFI to the required volume.

Administration

The formulations of the present invention are useful as a medicament. For example, in some embodiments, the formulations of the present invention are used for boosting the immune system of a patient after chemotherapy, raising white blood cell counts, and/or for improving the immune response to peptide vaccines. Thus, in some embodiments, the formulations of the present invention are for use in the treatment and/or prophylaxis of a patient, particularly a human. In some embodiments, the formulation is for use as an immune-modulatory agent.

The formulation may be used in the treatment and/or prophylaxis of cancer. In some embodiments, the cancer is one or more of adrenal gland, autonomic ganglia, biliary tract, bladder, bone, breast, central nervous system, cervical, colorectal, endometrial, haematopoietic,

lymphoid, kidney, large intestine, liver, lung, non-small cell lung, oesophagus, ovarian, pancreatic, prostate, salivary gland, skin, small intestine, stomach, testicular, thymus, thyroid, upper aerodigestive tract and urinary tract cancer and/or malignant melanoma. In some embodiments, the cancer is colorectal, lung and pancreatic cancer. In some embodiments, the cancer is cancer that arises due to a mutation in the RAS protein.

The formulations of the present invention may also be used for the reduction of neutropenia in patients receiving chemotherapy. A further use of the formulations of the present invention is to accelerate myeloid recovery in patients following bone marrow transplant, or for the treatment of super-orphan lung diseases such as pulmonary alveolar proteinosis (PAP).

In some embodiments, the use of the formulations of the present invention as a medicament comprises use with a peptide vaccine. Such use is particularly useful for the treatment and/or prophylaxis of cancer. In some embodiments, the peptide vaccine is a RAS peptide vaccine, which is used for the treatment and/or prophylaxis of cancer arising due to a mutation in the RAS protein, such as RAS peptide vaccines described in WO2015/086590, WO2015/169804, and WO2016/202937, incorporated herein by reference. In other embodiments, the peptide vaccine is as described in WO00/02581, which is incorporated herein by reference. In some embodiments, the formulations of the present invention are for use simultaneously or sequentially with a peptide vaccine. When administered sequentially, the formulation of the present invention and the peptide vaccine are administered one after another. The time between administration of the formulation and the peptide vaccine may be about 5, 10, 15, 20, 25 or 30 minutes. In other embodiments, the formulation and the peptide vaccine are administered simultaneously. Simultaneous administration may involve administering the formulation and the peptide vaccine one immediately after the other, with no, or substantially no, delay between the administration of the formulation and the peptide vaccine. Alternatively, simultaneous administration may involve administrating both the formulation of the present invention and the peptide vaccine as a single dose, for example, by a single injection.

Formulations are prepared for administration to a patient by reconstituting the lyophilised formulation in sterile WFI, according to procedures known in the art. For example, in one method of reconstitution of the formulation of the invention, one vial of the lyophilised formulation containing 0.1mg of the GM-CSF protein is reconstituted with 0.33ml of sterile WFI, to give a concentration of the GM-CSF protein of 0.3mg/ml. The date and time of reconstitution is recorded on the vial label, and the vial is gently rotated (to minimise foaming). The vial is allowed to rest for 10 minutes before preparation of a syringe for administering the formulation.

The reconstituted formulation is inspected for visible particle, and is rotated again if any particles are visible. The vial is then allowed to rest for a further 10 minutes, after this second rotation. (If particles are still visible after the second rotation, then the vial is not used and is be reported as an IMP deviation.) The reconstituted formulation is stored at 2-8°C, away from sunlight, and is used within 6 hours of reconstitution. In some instances, the reconstituted formulation is stored at 2-8°C, away from sunlight, for up to 12 hours before use.

The formulations of the present invention may be administered to the patient by any suitable delivery technique known to those skilled in the art. For example, among other techniques, the formulation of the present invention may be administered to a subject by intradermal injection, subcutaneous injection, intra-muscular injection, intravenous infusion, or nasal administration or inhalation.

Examples

Example 1

1. Scope and Objectives

The purpose of this study was to define promising formulation candidates for the development of a liquid or lyophilised GM-CSF drug product for intradermal application. An existing formulation (20 mM K-phosphate, pH 7.3) of GM-CSF drug substance (GM-CSF Control Sample 1) served as the basis for the development. Suitable buffer systems and excipients were selected and tested with respect to their relevance for increasing the colloidal as well as the thermodynamic stability of GM-CSF.

The following actions were performed to execute this study:

- Based on GM-CSF Control Sample 1 (20 mM K-phosphate, pH 7.3), two designs of experiment (DoE) test matrices targeting the main effect of pH variation, buffer composition and ionic strength on the thermodynamic and colloidal stability of GM-CSF were designed. Preparation and analysis of all variants of the test matrices was performed in order to measure the predictable thermodynamic and colloidal stability by means of nanoDSC and CG-MALS, respectively.
- A selection of ionic and non-ionic stabilizers was tested for their relevance of stabilizing the active pharmaceutical ingredient (API) further thereby also adjusting the formulation candidates to isotonicity.

2. Materials and Apparatus

2.1 Substances and Materials

Table 1 – Materials and substances

Substances	Lot no.:	Manuf. / Suppl.	Order no.:
GM-CSF Control Sample 1 (SEQ ID NO: 3)	150904/1064/71	-	-
GM-CSF Control Sample 2 (SEQ ID NO: 3)	160126/1089/082	-	-
ortho-phosphoric acid 85 %, EMPROVE® exp. Ph.Eur	K47168363542	Merck Chemicals	100563
Sodium chloride, EMPROVE®exp Ph. Eur., BP, USP	K93205800	Merck Chemicals	106400
L-histidine, Ph. Eur.	134211300	Carl Roth GmbH	3852.2
L-arginine, ≥ 98.5 %, USP	266238404	Carl Roth GmbH	3144
L-proline, ≥ 98.5 %, Ph.Eur.	076233849	Carl Roth GmbH	T205
Sucrose, EMPROVE® exp. Ph.Eur	K42784453205	Merck Chemicals	107653
D(-)-Mannit, ≥ 98 %, Ph.Eur., USP	355233673	Carl Roth GmbH	4175.1
Glycerol, 98 %, Ph.Eur, anhydrous	146242100	Carl Roth GmbH	7530
Citric acid, anhydrous, Ph.Eur	265230599	Carl Roth GmbH	6490.1
TRIS, ≥ 99 %, Ph.Eur., USP	173197442	Carl Roth GmbH	A411
Hydrochloric acid 25 %, EMPROVE® exp. Ph Hely	Z0345812517	Merck Chemicals	100312
Potassium chloride, 99 %, Ph.Eur., USP, BP	141168919	Carl Roth GmbH	P017
Sodium hydroxide pellets, EMPROVE®exp Ph.Eur.	81126182508	Merck Chemicals	106482
Potassium dihydrogen phosphate, 98 %, Ph.Eur., USP, BP	261168601	Carl Roth GmbH	P018
di-Sodium hydrogen phosphate dihydrate, for analysis EMSURE® Ph.Eur.	K43038880 201	Merck Chemicals	106580
MES - Running buffer (20x)	-	Invitrogen	NP0002
Methanol, ROTISOLV HPLC Gradient	114A0214170958	Carl Roth GmbH	KK39
NuPAGE LDS Sample Buffer	1771449	Invitrogen	NP0007
Precision Plus MW-Marker	-	Biorad	161-0374
SimplyBlue Safestain	1769355	Invitrogen	46-5034
Materials			
Slide-A-Lyzer dialysis cassettes, 15 mL; MWCO: 3.5 kDa	00600977	Thermo Scientific	87724
Minisart® PES 0.1 µm syringe filter	60395103	sartorius stedim	16553-K

Acquity UPLC Protein BEH SEC, 1.7 µm, 150 x 4.6 mm	0226360741* & 0245362081**	Waters GmbH	186005225
11 mm crimpsnap-vial, polypropylene, 500 µL	00223149	WICOM Germany GmbH	WIC 4720
12 % Bis-Tris NuPAGE Gel, 1mm x 12 well		Invitrogen	NP0342PK2
1.5 mL reaction vessel	F167398K	Eppendorf	0030.120.086

2.2 Apparatus

The following apparatus and equipment was used:

Purified water supply

- Manufacturer: Siemens
- Type: Ultra Clean UV OF TM

Static light scattering detector

- Manufacturer: Wyatt Technology Corporation (Santa Barbara, CA, USA)
- Type: Heleos

UV Spectrophotometer

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: Cary 60 UV-Vis
- SN: MY16170003

CG-MALS

- Manufacturer: Wyatt Technology Europe (Dernbach, Germany)
- Type: Calypso II
- Software: Calypso Version 2.1.3

nanoDSC

- Manufacturer: TA instruments (New Castle, DE, USA)
- Type: nanoDSC

HPLC Equipment

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: 1100 Series
- Degasser G1322A
- BinPump G1312A
- ALS G1329A
- ALSTherm G1330A
- ColCom G1316A
- DAD G1315A

Electrophoresis

- Manufacturer: Thermo Fisher Scientific
- Type: Novex XCell SureLock Electrophoresis Cell
- Power supply: Consort EV265, Hoefer Inc

Additional Laboratory Equipment

- Analytical balance (Kern)
- Magnetic stirrer (IKA Werke)
- pH meter (Mettler Toledo)
- Volumetric pipets (Gilson)

3. Methods

3.1 Nano Differential Scanning Calorimetry (nanoDSC)

Nano DSC is specifically designed to determine the denaturation temperature and thermal denaturation enthalpy of proteins and other macromolecules in solution with the versatility and precision to perform molecular stability screenings.

Experiment method:

Mode: Scanning

Temperature parameters: Lower: 20 °C
Upper: 100 °C
Rate: 1 °C/min heating and cooling
Equilibration: 600 s

Pressure parameters: Manual, 3.0 atm

Data interval: 1s

A buffer scan was conducted prior to each sample run to generate a baseline.

Sample preparation:

Samples were dialyzed against the corresponding formulation buffer (see 3.4) and diluted to 1mg/mL. The corresponding dialysis buffer was used as buffer scan and buffer reference.

Interpretation of analysis data:

Differential scanning calorimetry measures the amount of heat that is absorbed or released from biomolecules in solution during heating or cooling. Native proteins respond to heating by unfolding (thermal denaturation) at a characteristic temperature (Tm). The more intrinsically stable the biopolymer is the higher is the onset temperature of the unfolding transition and the transition enthalpy (H). The onset temperature of thermal denaturation, i.e. the starting point of the unfolding transition, is defined as T_{onset}.

3.2 Composition gradient multi-angle light scattering (CG-MALS)

Interactions between protein molecules in solution were characterized by changes in their light scattering behaviour at different concentrations via MALS. With this series of light scattering measurements the second virial coefficient A_2, which is a characteristic parameter to evaluate molecule interactions, was calculated.

A_2 is characteristic for a substance and its solvent, and describes molecular interactions between the dissolved components. A negative A_2 indicates attractive interactions between molecules of the dissolved substance whereas a positive A_2 is characteristic for repulsive interactions between the dissolved protein molecules (Valent *et al.* "Colloidal behaviour of proteins: Effects of the second virial coefficient on solubility, crystallization and aggregation pf proteins in aqueous soluntion", Current Pharmaceutical Biotechnology, 2005, 6, 427-436).

The CG-MALS technique is based on a series of differently concentrated macromolecular solutions which are directly injected into the flow cell of a multi-angle light scattering detector. After each injected concentration step the flow is stopped to permit the reaction to reach equilibrium for signal stabilization and subsequent detection. The apparent weight average molecular weight (Mw_{app}) is determined for each step in the concentration gradient by analyzing the light scattering and concentration data by the following equation:

$$Mw_{app} = \frac{R(\theta,c)}{K \times c}$$

Mw_{app}: apparent molecular weight

R(θ,c): excess Rayleigh ratio of the solution as a function of scattering angle 8 and concentration c. It is directly proportional to the intensity of the excess light scattered by the solute and the light scattered by the pure solvent.

K: Optical constant $(4p^2(dn/dc)^2n_0^2/N_al_0^4)$

c: Concentration

Significant interactions between macromolecules manifest as changes in Mw_{app} vs. concentration.

A_2 calculation was conducted via Zimm plot analysis by an extrapolation to 0 mg/mL concentration according to:

$$\frac{K^* c}{R(\theta,c)} = \frac{1}{M_w P(\theta)} + A_2 c$$

R(θ,c): excess Rayleigh ratio of the solution as a function of scattering angle θ and concentration c. It is directly proportional to the intensity of the excess light scattered by the solute and the light scattered by the pure solvent.

M_w: Weight average molecular weight

A_2: 2nd virial coefficient

c: Concentration

K^*: Optical constant $(4p^2(dn/dc)^2n_0^2/N_aI_0^4)$

P(θ): describes the angular dependence of the scattered light, and can be related to the rms radius

Sample preparation:

Samples were dialyzed against the corresponding formulation buffer (see part 3.4) and used undiluted. The corresponding dialysis buffer was used as diluent for the CG-MALS experiment. The samples were passed over a 0.1 µm syringe filter. Before loading the sample into the CG-MALS system the exact concentration of the sample was determined by UV-absorption measurement (see part 3.3). Sample and buffer were loaded into the Calypso II system and the measurement was started.

Sample measurement:

A Calypso II CG-MALS system was used to supply the MALS detector with the concentration gradient of the analyte. The sample was loaded on one syringe pump of the system and the dialysis buffer on another syringe pump of the system. Ten equidistant concentration steps from approx. 3.5 mg/mL to 0.5 mg/mL were applied in each measurement by diluting the sample with dialysis buffer. At each gradient step 1 mL of sample was injected into the MALS detector. The resulted light scattering signal was recorded over a time period of 180 seconds. In addition to the light scattering signal the concentration of each gradient step was recorded using a refractive index detector. Control of the system and analysis of the data via Zimm plot analysis was performed with Calypso software version 2.1.5.

3.2.1 Binary CG-MALS measurements

The two protein samples of interest were loaded on syringe pump 1 and syringe pump 2 of the system and the dialysis buffer was loaded on syringe pump 3. The binary CG-MALS measurement consists of three steps. In the first step, a concentration gradient of sample 1 ranging from 10 % to 100 % was applied to determine the self-virial coefficient of sample 1 in the formulation. The second step consists of a cross-over gradient, in which the concentration of sample 1 was reduced from 90 % to 10 % while the concentration of sample 2 was increased

from 10 % to 90 % in ten steps. This step was conducted to determine the cross-virial coefficient. In the third step a concentration gradient of sample 2 ranging from 100 % to 10 % was applied to determine the self-virial coefficient of sample 2 in the formulation. Figure 1 displays schematically the steps of a binary CG-MALS experiment to detect the self-virial coefficient of the two molecules as well as the cross-virial coefficient which measures the interactions between the two protein species. At each gradient step 1 mL of sample was injected into the MALS detector. Zimm plot analysis was performed with Calypso software version 2.1.5.

3.3 UV Measurement

The concentration of GM-CSF in solution was determined using a Cary 60 UV-spectrometer (Agilent Technologies, Santa Clara, USA) by adsorption measurement at 280nm. Samples were measured at a concentration of about 1mg/mL using plastic cuvettes with an optical path thickness of 1.0cm. The concentration was calculated according to Lambert-Beer's law using an extinction coefficient of 0.974mL/(mg*cm) as provided by Wacker Biotech for GM-CSF.

3.4 Dialysis

The dialysis of GM-CSF was accomplished in three independent dialysis steps to achieve a quantitative buffer exchange. 8 mL of the GM-CSF drug substance (c = 3.5 mg/mL) was transferred into preconditioned (in dialysis buffer) Slide-A-Lyzer cassettes (Thermo Scientific, Rockford, USA). Filled Slide-A-Lyzer cassettes were incubated in 650 mL of the target buffer for 2 hours before a first buffer change (650 mL) was performed. After dialysis for two further hours the buffer was changed a second time (650 mL) to finalize the dialysis overnight. The protein sample was removed from the Slide-A-Lyzer cassettes and processed further. Each dialysis step represents a 1/80 fold buffer exchange leading to a calculated buffer exchange factor of 5 x 10^5 in total.

4. Results and Discussion

The predictive formulations analytics comprised a screening experiment consisting of variants with different pH, buffer components, and additional stabilizers to identify suitable formulation conditions for GM-CSF with respect to thermodynamic (measurement by nanoDSC) and colloidal stability (measurement by CG-MALS).

The pH was modulated from 6.0 to 8.2 in order to accommodate an intradermal administration close to physiologic pH. For each pH value, suitable buffer components were selected to achieve a sufficient buffer capacity. The buffer components were selected from commonly used

pharmaceutically accepted substances. As buffer components L-histidine/HCl, citric acid/NaOH, potassium phosphate and Tris/HCl were selected. Potassium phosphate buffer was used because the GM-CSF drug substance (DS) was supplied in it by default and also as it covers a broad pH range. L-histidine/HCl is often used for antibody formulations and covers neutral to slightly acidic pH ranges. Citric acid/NaOH was chosen as an intermediate buffer system between His/HCL and potassium phosphate. Tris/HCl is active at slightly basic pH values due to its basic pKs. During the first pre-screening (part 4.1) only the pH and buffer systems were varied.

To investigate the impact of high and low ionic strength as well as of high and low buffer concentration, formulations with and without addition of potassium chloride and, respectively, with high and low buffer molarity were analyzed in the first DoE matrix (part 4.2).

Additionally, formulations were supplemented with potential protein stabilizers and bulking agents in the second DoE matrix (part 4.3) to detect any beneficial effects of these excipients.

4.1 Pre-Screening

The results of the pre-screening with different buffering agents covering a pH range from pH 6.0 to pH 8.2 are shown in Table 2.

Table 2 - Results of the pre-screening.

variant #	pH	buffer system (10 mM)	A2 [mol x mL x g-2 x 10^{-4}]	T_{onset} [°C]	T_M [°C]	deltaH [KJ/mol]
1	6.0	His/HCl	-15.3	39.7	43.3	145
2	6.4	Citric acid/NaOH	-8.9	51.7	60.0	159
3	7.2	K-Phosphate	-2.8	50.9	60.3	204
4	8.2	Tris/HCl	> 1	50.2	58.9	223

Variant #1 showed some strong aggregation of insoluble aggregates visible after dialysis, a consolidated precipitation was observed.

The A_2 of all variants besides #4 (basic pH) was measured as a clear negative value, indicating strong attractive interactions. Neutral interactions would be shown by values around 0, clearly repulsive interactions would be shown by positive values clearly above zero. Such a positive A_2 value was obtained only for variant #4, whereas a really noisy signal was observed (this could also be re-confirmed in an additional reproduction run). It was not so obvious to define a definite value for #4, but it can be noted that always clearly positive values were calculated (indicated by

">1" in Table 2). Further, a trend could be observed towards more repulsive interactions (towards more positive A_2 values) with increasing pH (towards neutral and above towards basic pH).

The T_{onset} (the onset temperature of thermal denaturation, i.e. the starting point of the unfolding transition) of variants #2 - #4 (neutral towards basic pH) is above 50 °C and quite distant from any commonly used storage and handling temperature, thus lies in the non-critical range. T_{onset} of pH 6.0 variant #1 is clearly lower towards more critical range. The positive effect of increasing pH on protein stability is also confirmed by continuously increasing enthalpies ΔH for thermal denaturation. An exemplary thermogram is shown in Figure 2.

4.2 First DoE matrix

Based on these findings of the pre-screening it could be confirmed that neutral to basic pH (e.g. K-phosphate and Tris buffer) seemed to be beneficial for stabilizing GM-CSF. Thus, the first DoE matrix was designed around K-phosphate buffer (which is known to be a suitable buffer for GM-CSF) at differing pH, ionic strength, and buffer molarity. The results are listed in Table 3.

Table 3 - Results of the first DoE matrix. K-phosphate buffer served as buffer constant for the matrix.

#	pH	Ionic stabilizer [mM] (KCl)	Buffer molarity [mM]	A2 [mol x mL x g^{-2} x 10^{-4}]	T_{onset} [°C]	T_M [°C]	Delta H [kJ/mol]
5	7.3	0	10	0.5	51.3	59.4	157.2
6	7.3	150	10	2.6	55.3	65.0	254.2
7	7.3	0	50	0.5	55.5	63.5	113.8
8	7.7	75	30	-3.7	55.6	64.2	205.4
9	7.7	75	30	-1.6	55.8	64.9	196.6
10	8.1	0	10	18.4	51.8	61.4	197.5
11	8.1	150	10	-3.6	55.2	65.3	214.4
12	8.1	0	50	0.4	54.8	64.9	224.4

As the signal was again determined to appear very noisy, the numerical A_2 values are to be regarded as approximate.

At pH 7.3 high ionic strength conditioned a clearly positive A_2 (repulsive interactions, see #6), while at pH 8.1 low ionic strength was more beneficial (#10). Thus, most likely an effect of ionic strength could be attributed to the A_2 value, which however was influenced strongly by pH. The

closer a formulation variant's pH was to the isoelectric point (pI) of the molecule (= 5.2), the more positive was the influence of a higher ionic strength towards repulsive interactions. Most prominently this could be seen between variants #5 and #6.

The more negatively charged the molecule was towards basic pH, the more this effect of high ionic strength sloped, and even started to have a negative impact on the A2. This was visible between variants #10, #11 and #12.

Also, at the centre point formulations (#8, #9), the higher ionic strength compared to e.g. #5 and #10 already seemed to have a negative effect.

The values for T_{onset} confirm these results partly. Variant #10 together with #5, both featuring lowest ionic strength within the matrix, showed the lowest values (~ 51 °C). All other variants were comparable in their T_{onset} values(~ 55 °C).

A statistical evaluation of the first DoE matrix confirms the observations summarized above. The statistical evaluation resulted in the effects for the A2 and the T_{onset} shown in Figures 3 and 4.

For the A_2, it can be seen that an increase of the pH from pH 7.3 to pH 8.1 shows in tendency a positive effect. Furthermore, the addition of salt has a negative effect, mainly caused by the strong decrease of A_2 at basic pH when salt is added. The buffer molarity acts comparably to the addition of salt.

For the T_{onset}, it can be seen that an increase of the pH shows no effect. Furthermore, the addition of salt has a positive effect independent of the pH. The buffer molarity acts comparably to the addition of salt.

4.3 Second DoE matrix

The second DoE matrix was designed to test stabilizing effects of several excipients on GM-CSF (variants #13 - #21). A combination of excipients suitable for liquid and/or lyo formulations was selected to keep both options open, which decision will be subject to chemical stability data that will be obtained in a forced degradation study. Variant #5 was selected as a root variant facilitating a liquid and freeze-dried presentation, variant #6 was selected as preferred root variant for a liquid DP presentation only, and variant #10 was selected as a preferred root variant for a lyophilized DP presentation. Excipient concentration was selected to facilitate isotonicity (~300 mM). In addition, the potential effect of a different buffering agent was

evaluated by interchanging K-phosphate with Tris/HCl as this buffer showed a positive effect in the pre-screening already (variant #22, variant #23). Table 4 lists the variants tested during the excipients screening and the respective results.

Table 4 - results of the second DoE matrix, excipients screening. Root variants were: #5 10mM K-phosphate pH7.3; #6 10mM K-phosphate 150mM KCl pH7.3; #10 10mM K-phosphate pH8.1

#	buffer	comment	A_2 [mol x mL x g^{-2} x 10^{-4}]	T_{onset} [°C]	T_M [°C]	Delta H [kJ/mol]
13	10 mM K-phosphate, pH 7.3, 260 mM sucrose	liquid and lyo (root variant #5)	-2.8	n.d.	n.d.	n.d.
rep. 13	10 mM K-phosphate, pH 7.3, 260 mM sucrose	liquid and lyo (root variant #5)	-4.5	54.3	61.6	413.9
14	10 mM K-phosphate, pH 7.3, 260 mM arginine / H_3PO_4	liquid and lyo (root variant #5)	-2.1	56.0	66.0	302
15	10 mM K-phosphate, pH 7.3, 260 mM mannitol	liquid and lyo (root variant #5)	-2.5	52.0	62.0	293
16	10 mM K-phosphate, pH 7.3, 260 mM glycerol	Liquid only (root variant #5)	-2.0	n.d.	n.d.	n.d.
17	10 mM K-phosphate, pH 7.3, 260 mM L-proline	Liquid only (root variant #5)	-2.1	52.0	61.0	279.0
18	10 mM K-phosphate, pH 7.3, 150 mM NaCl	Liquid only (root variant #6)	-2.4	56.0	65.0	237.0
19	10 mM K-phosphate, pH 8.1, 260 mM sucrose	Lyo only (root variant #10)	2.3	54.0	64.0	251.0
20	10 mM K-phosphate, pH 8.1, 260 mM arginine/ H_3PO_4	Lyo only (root variant #10)	1.2	57.0	66.0	196.0
21	10 mM K-phosphate, pH 8.1, 260 mM mannitol	Lyo only (root variant #10)	1.5	53.0	62.0	164.0
22	10 mM Tris/HCl, pH 7.3, 260 mM sucrose	liquid and lyo (root variant	-0.8	50.4	60.0	n.d.
23	10 mM Tris/HCl, pH 8.1, 260 mM sucrose	Lyo only (root variant #19)	11.9	49.7	59.8	335.10

Excipients have been selected for the following reasons:

- Arg/Phosphate: ionic strength, bulking agent, protein stabilizer, stability and solubility enhancer
- Sucrose: stabilizer
- Mannitol: non-ionic protein stabilizer and bulking agent
- Glycerol: non-ionic protein stabilizer, a smaller polyol, for liquid only, cannot be freeze-dried
- Proline: hydrotropic substance with aggregation protection effects e.g. also applied for protein refolding, but cannot be lyophilized as it does not provide a stable lyo matrix

Excipient screening for root variant #5 (variants #13 - #17) did not improve the A_2 towards more repulsive interactions, but rather shifted the tendency towards attractive interactions in all variants (lower colloidal stability). However, with regard to T_{onset}, T_M, and deltaH, the thermodynamic stability could be enhanced by the addition of excipients in all variants. For variants #13 and #16, no evaluation of the nanoDSC data (thermodynamic stability) was possible (ambiguous thermograms were obtained). After repetition, the nanoDSC data could be evaluated for variant #13 (see Table 4, rep. 13), but not for variant #16.

Unexpectedly, excipient screening using 150 mM NaCl in variant #18 (instead of 150 mM KCl in #6) did shift the A_2 towards attractive interactions (lower colloidal stability). Thermodynamic stability remained more or less unchanged, or rather decreased slightly by addition of NaCl.

Excipient screening for root variant #10 (variants #19 - #21) revealed still clear repulsive interactions, even if the A_2 values were lower compared to variant #10. As the A_2 value does not follow a linear correlation, values above 0.5 in the positive range can be regarded indicative for repulsive interactions - thus, facilitating colloidal stability. Variant #19 also showed a distinct increase in unfolding enthalpy, whereas variant #21 showed decreased enthalpy values. All variants #19 - #21 showed a slight tendency towards a higher T_{onset} and T_m, thus a higher thermodynamic stability, accounting for the fact that the addition of excipients did influence the thermodynamic stability of root variant #10 positively.

Overall, it could be observed that the excipients within a measurement row (variants #13 - #17, and #19 - #21) did not have distinct effects amongst each other. Only T_{onset} and T_m seem to be beneficially influenced by the presence of L-arginine/H_3PO_4 (#14 and #20).

The positive effect on the A_2 in variants #19 - #21 can most likely be attributed to the high basic pH.

Measurements #22 and #23 were performed to evaluate the effect of a different buffering agent while keeping pH and excipient constant. As seen in K-phosphate buffer (#13, #19), A_2 values at a pH of 7.3 indicate attractive interactions (negative value), and at basic pH 8.1 clear repulsive interactions.

For variant #22, however, the A_2 value could be shifted clearly towards less attractive interactions, almost neutral interactions. For variant #23 a high A_2 value of 11.9 was measured which was the highest A_2 value measured so far on the API.

However, by changing from K-phosphate to Tris/HCl buffer, the thermodynamic stability (T_{onset} and T_m) dropped some distinct °C units, which is less favourable. The Tris/HCl buffer thus represents a compromise between more beneficial colloidal stability (A_2) and unfavourable thermodynamic stability.

As Tris/HCl did not outperform the K-phosphate buffer in all instances, the recommendation for the subsequent binary rHA measurements (third DoE matrix) was to use the K-phosphate buffer.

Conclusion:

Based on the overall results of the second DoE, the lyo formulation variants #19 - #21 seem to present the more stable formulations for GM-CSF with regard to maximizing colloidal and thermodynamic stability at the same time (highest value for A_2, highest value for T_{onset}) - any of the tested isotonic liquid/lyo or liquid formulations would always account for a compromise between higher thermodynamic stability, but lowered colloidal stability.

5. Conclusions

In this study, the thermodynamic properties and colloidal stability of GM-CSF in different formulation variants were evaluated. By varying pH, buffering agents, ionic strength, and excipients the most beneficial variants for either lyo or liquid formulation were determined successively.

In all GM-CSF measurements, the signal to noise ratio was very low. Nonetheless, the following results can be summarized:

Pre-screening:

- Screening of pH between 6.0 and 8.2 showed a beneficial effect of more basic environment on colloidal stability (positive A_2)
- GM-CSF in His/HCl buffer at pH 6.0 showed poor thermodynamic properties, whereas all other tested systems showed acceptable results with T_{onset} around 50 °C.

First DoE matrix:

- Higher ionic strength seemed to have a beneficial effect on the A_2 at pH 7.3 but shows the opposite effect at pH 8.1.
- The thermodynamic properties, however, seem to benefit from higher ionic strength in every scenario.

Second DoE matrix:

- None of the excipients had a huge effect on either colloidal stability or thermodynamic properties. Merely the addition of NaCl instead of KCl had a truly negative effect on the A_2 value.
- Champion formulations for subsequent measurements were 10 mM K-phosphate with 260 mM sucrose at pH 7.3 and pH 8.1, respectively.

The following 4 test variants (Table 5) are evaluated further in a forced degradation study (see Example 2).

Table 5 - Test variants to be evaluated further in a forced degradation study.

Test Variant #	Formulation variant	Comment
1	10 mM K-phosphate, pH 7.3, 260 mM sucrose (#13)	Liquid and lyo
2	10 mM K-phosphate, pH 7.3, 260 mM Arginine/H_3PO_4	Liquid and lyo
3	10 mM K-phosphate, pH 8.1, 260 mM sucrose (#19)	Lyo only
4	10 mM K-phosphate, pH 8.1, 260 mM Arginine/H_3PO_4	Lyo only

Sucrose and arginine/H_3PO_4 seemed suitable excipients to stabilize the API - T_{onset} and A_2 were amongst the highest. Mannitol as an additional bulking agent might still be added to form a proper lyo cake (to be determined during lyo cycle test and development). Both pH values of 7.3 and 8.1 would be suitable for a lyophilized drug product (DP) and thus might be evaluated further.

Example 2

1. Scope and Objective

The aim of this study was to find the most promising formulation variants for a GM-CSF lyophilized drug product by applying five different harsh stress conditions and subsequent analysis with regard to aggregate status and potential chemical degradation. The two most promising formulation candidates were selected for further development and analysis. Based on the outcome of Example 1, above, the four test variants displayed in Table 6 were selected for this study.

Table 6: Test variants for the GM-CSF forced degradation study

Test Variant #	Formulation	Comment
1	10 mM K-phosphate, 260 mM sucrose, pH 7.3	Liquid and lyo
2	10 mM K-phosphate, 260 mM Arginine/H3PO4, pH 7.3	Liquid and lyo
3	10 mM K-phosphate, 260 mM sucrose, pH 8.1	Lyo only
4	10 mM K-phosphate, 260 mM Arginine/H3PO4, pH 8.1	Lyo only

The test variants were prepared with a GM-CSF target concentration of 0.3 mg/mL and spiked with purified water (w/o), polysorbate 20 (PS20), poloxamer 188 (P188), and recombinant human albumin (rHA), respectively, in order to evaluate the effect of these additives on the stability of GM-CSF. Furthermore, these resulting 16 sub-variants were enclosed in lyo vials with either air atmosphere or nitrogen atmosphere, respectively, resulting in a total of 32 sub-variants. In addition to the described variants, a “control” forced degradation run was performed using a reconstituted lyophilizate of an existing commercial formulation of GM-CSF (“current DP”), containing human serum albumin (HSA), mannitol, macrogol 4000, dibasic sodium phosphate and monobasic potassium phosphate.

2. Materials and Apparatus

2.1 Substances and Materials

Table 7 - Materials and substances used in the forced degradation studies

Substances	Lot no.:	Manuf. / Suppl.	Order no.:
GM-CSF Control Sample 1 (SEQ ID NO: 3)	150904/1064/71	-	-

GM-CSF Control Sample 2 (SEQ ID NO: 3)	160126/1089/082	-	-
Existing commercial formulation lyophilizate (“control”)	A201501A02	-h	-
BEH200 SEC Protein Standard Mix	-	Waters GmbH	186006518
Recombumin® Alpha (20 % (w/v))	RF20-005	Albumedix	230-010
Kolliphor® P 188, Poloxamer, Ph. EUR.	WPDH566B	BASF	50259527
Tween® 20, EMPROVE® exp Ph. Eur. (polysorbate 20)	K47935972	Merck KGaA	817072
Albumin, Monomer bovine, Monomer ≥	SLBH7604V	Sigma-Aldrich	A1900
ortho-phosphoric acid 85 %, EMPROVE® exp. Ph. Eur.	K47168363542	Merck Chemicals	100563
Sodium chloride, EMPROVE® exp Ph. Eur. BP, USP	K45515600	Merck Chemicals	106400
L-arginine, ≥ 98.5 %, USP	266238404	Carl Roth GmbH	3144
Sucrose, EMPROVE® exp. Ph.Eur	K42784453205	Merck Chemicals	107653
TRIS, ≥ 99 %, Ph.Eur., USP	173197442	Carl Roth GmbH	A411
Hydrochloric acid 25 %, EMPROVE® exp. Ph Helv	Z0345812517	Merck Chemicals	100312
Sodium hydroxide solution, 30 % w/w pure, pharma grade	0000862568	AppliChem	144320.1211
Potassium hydroxide, EMPROVE® exp Ph Eur	B03389302	Merck Chemicals	105032
Potassium dihydrogen phosphate, ≥98 %. Ph.Eur.. USP. BP	261168601	Carl Roth GmbH	P018
di-Sodium hydrogen phosphate, ≥98 %, Ph.Eur	373201802	Carl Roth GmbH	T876
Trifluoroacetic acid, ≥ 99.9 %, for peptide synthesis	245230492	Carl Roth GmbH	P088.1
MES – Running buffer (20x)	-	Invitrogen	NP0002
Acetonitrile, Chromasolv gradient grade	SZBG041AV	Sigma-Aldrich	34851
NuPAGE LDS Sample Buffer	1771449	Invitrogen	NP0007

Precision Plus MW-Marker	-	Bio-Rad	161-0374
SimplyBlue Safestain	1769355	Invitrogen	46-5034
Materials			
Slida-A-Lyzer dialysis cassettes, 15mL; MWCO: 3.5 kDa	00600977	Thermo Scientific	87724
Minisart® PES 0.1 µm syringe filter	60395103	Sartorius stedim	16553-K
Jupiter 5 µm C18 (300 Å; 250 x 4.6 mm)	H16-035225	Phenomenex	00G-4053-E0
ProPac WAX-10 (4 x 250 mm)	01531083	Thermo Scientific	054999
Superdex™ 200 Increase 10/300 GL	10246668	GE Healthcare	28-990o9-44
Acquity UPLC Protein BEH SEC, 1.7 µm, 150 x 4.6 mm	0226360741 & 0245362081	Waters GmbH	186005225
11 mm crimpsnap-vial, polypropylene, 500 uL	00223149	WICOM Germany GmbH	WIC 4720
12 % Bis-Tris NuPAGE Gel, 1 mm x 12	-	Invitrogen	NP0342PK2
1.5 mL reaction vessel	F167398K	Eppendorf	0030.120.086
2R FIOLAX injection vials, clear	162212007	Nipro Germany	-
Lyo stopper, 1356 4023/50/GREY B2-TR Westar®RS SQS	1152012315	West	7002-3047
Serum stoppers, 1256 4110/40/GREY, silicone 1/4 A	1152062780	West	7000-3823
Flip off crimp caps, green and red	-	PharMediPack	-

2.2 Apparatus

The following apparatus and equipment was used:

Purified water supply

- Manufacturer: Siemens
- Type: Ultra Clean UV UF TM

UV Spectrophotometer

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: Cary 60 UV-Vis
- SN: MY16170003

HPLC Equipment

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)

- Type: 1100 Series
- Degasser G1322A
- BinPump G1312A
- ALS G1329A
- ALSTherm G1330A
- ColCom G1316A
- DAD G1315A

Electrophoresis

- Manufacturer: Thermo Fisher Scientific
- Type: Novex XCell SureLock Electrophoresis Cell
- Power supply: Consort EV265, Hoefer Inc

Suntest

- Manufacturer: Atlas Material Testing Technology (Linsengericht-Altenhasslau, Germany)
- Type: Suntest CPS+

Pilot freeze dryer (GT2)

- Manufacturer: Hof Sonderanlagenbau (Lohra, Germany)
- 0.36 m2 shelf area
- In-vial temperature recording
- Differential pressure measurement

Additional Laboratory Equipment

- Analytical balance (Kern)
- Magnetic stirrer (IKA Werke)
- pH meter (Mettler Toledo)
- Volumetric pipets (Gilson)
- Multistep Pipettes: Brand, HandyStep
- Autoclave: Systec, DX65
- Drying oven: Memmert, 500
- Orbital shaker: Wisd Laboratory Instruments, WiseShake SHO-1D
- Climate chamber: Binder
- Laminar flow microbiological class 2 work bench: BDK, UVF 6.18S

3. Methods

3.1 Forced degradation study

3.1.1 Preparation of "control"

In order to create a reference point for the results of the forced degradation study of the 32 sub-variants, a control run was performed using the "current DP" as reference formulation (an existing commercial formulation; composition as listed in section 1 "Objectives"). Therefore, "control" lyophilizates (0.1 mg/vial of GM-CSF) were reconstituted with 330 µL of water for injection (WFI) and pooled up to a total volume of 700 µL (hereinafter described as "control"). Subsequently the solution was filled into a 2R glass vial, stoppered with a serum stopper, and crimped using a 13 mm crimp cap before the same stress procedure was applied to it as to the formulation variants (see section 3.1.2 for details).

3.1.2 Preparation of test variants

Sterilization of vials and stoppers:

2R glass vials were washed with purified water and subsequently depyrogenized and sterilized by dry heat at 300 °C for 2 hours. Stoppers were autoclaved at 134 °C for 10 minutes and subsequently dried at 70 °C for 8 hours.

Preparation of formulation buffers and detergent/stabilizer stock solutions:

Table 8: Preparation of formulation buffers and detergent/stabilizer stock solutions.

Solution	Composition	Preparation procedure
Test variant #1	10 mM K-phosphate, 260 mM sucrose, pH 7.3	1.15 g of 85% (w/w) ortho-phosphoric acid and 89.0g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.3 using potassium hydroxide and the volume was filled up to 1000mL with purified water.
Test variant #2	10 mM K-phosphate, 260 mM L-arginine, pH 7.3	1.15 g of 85 % (w/w) ortho-phosphoric acid were topped with 900mL of purified water before the pH was adjusted to 7.3 using potassium hydroxide. 45.3g of L-arginine were added and diluted. Subsequently the pH was adjusted to 7.3 using 85% (w/w) ortho-phosphoric acid and the volume was filled up to 1000mL with purified water.

Test variant #3	10 mM K-phosphate, 260 mM sucrose, pH 8.1	1.15 g of 85% (w/w) ortho-phosphoric acid and 89.0g of sucrose were diluted in 900mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
Test variant #4	10 mM K-phosphate, 260 mM L-arginine, pH 8.1	1.15 g of 85% (w/w) ortho-phosphoric acid were topped with 900 mL of purified water before the pH was adjusted to 8.1 using potassium hydroxide. 45.3g of L-arginine were added and diluted. Subsequently the pH was adjusted to 8.1 using 85% (w/w) ortho-phosphoric acid and the volume was filled up to 1000mL with purified water.
Polysorbate 20 stock solution	10 % (w/v) polysorbate 20	1 g of polysorbate 20 was filled up to 10mL total volume with purified water and stirred until complete homogeneity.
Poloxamer 188 stock solution	10% (w/v) poloxamer 188	1g of poloxamer 188 was filled up to 1 mL total volume with purified water and stirred until complete dissolution.
rHA stock solution	10% (w/v) Recombumin® Alpha	500µL of 20% (w/v) Recombumin® Alpha were mixed with 500µL of purified water.

Dialysis:

The dialysis of GM-CSF drug substance was accomplished in three independent dialysis steps to achieve a quantitative buffer exchange. 12 mL of the GM-CSF drug substance (c = 3.52 mg/mL) were transferred into preconditioned (in dialysis buffer) Slide-A-Lyzer cassettes with a molecular weight cut off of 3.5 kDa and a maximum fill volume of 15 mL (Thermo Scientific, Rockford, USA). Filled Slide-A-Lyzer cassettes were incubated in 1000 mL of the target buffer for 2 hours before a first buffer change (1000 mL) was performed. After dialysis for two further hours the buffer was exchanged a second time (1000 mL) to finalize the dialysis overnight. The protein sample was removed from the Slide-A-Lyzer cassettes and processed further. Each dialysis step represents a 1/83 fold buffer exchange leading to a calculated buffer exchange factor of $1:5.7\times10^5$ in total. All dialysis steps were performed at 2-8 °C.

Compounding and Filling:

After dialysis the GM-CSF solution was analyzed via UV absorption measurement at 280 nm in order to determine the GM-CSF concentration. The solution was then diluted to the target

concentration for the forced degradation study of 0.3mg/mL using formulation buffer. Subsequently the solution was spiked with the respective detergent/stabilizer stock solution to obtain a concentration of 0.1% (w/w) detergent/stabilizer. The respective test variant without detergent/stabilizer was spiked with the same amount of purified water.

The readily compounded formulations were sterile filtered using a 0.1 µm PES syringe filter and filled into sterile 2R glass vials by aliquots of 700 µL using a multistep pipette. Subsequently the vials for air/nitrogen exchange were partially stoppered with lyo stoppers and placed in a freeze dryer. Sub-variants to be evaluated with air atmosphere were stoppered with serum stoppers and crimped with 13 mm crimp caps. All compounding/filling steps were performed under a laminar flow bench to avoid microbial contamination of the product.

Air/nitrogen exchange:

Vials to be tested with nitrogen atmosphere were partially stoppered with 13 mm lyo stoppers and placed in a freeze dryer. The air in the freeze drying chamber was removed by applying a vacuum of 100 mbar and subsequent venting of the chamber with pure nitrogen. This procedure was carried out three times. After the third cycle the vials were closed under nitrogen atmosphere and a pressure of 900 mbar absolute. The vials were then unloaded of the freeze dryer and crimped with 13 mm crimp caps. A compilation of all tested sub- variants is provided in section 4, Table 19.

3.1.3 Stress conditions and sampling points

For sampling and analysis, the crimp caps and stoppers were removed from the respective vials before the formulation was transferred into 1.5 mL Eppendorf reaction vessels. From these vessels the respective volumes of samples were taken for the five different analytical methods: 60 µL for IEX-HPLC, 130 µL for SE-HPLC, 130 µL for RP-HPLC, 150 µL for UV absorption measurements, and 40 µL for SDS-PAGE. A compilation of all tested stress conditions and sampling points is provided in section 4, Table 20.

T=0

Sampling right after compounding and filling without any further stress.

Freeze/Thaw

Vials were placed in a freeze dryer in upright position and the program listed in Table 9 was applied.

Table 9: Lyo parameters for application of the freeze/thaw stress conditions

Step	Start temp [°C]	End temp [°C]	Scanning rate [°C/min]	Duration [min]
Loading	+25	+25	-	1

Freezing, ramp	+25	-50	1	75
Freezing	-50	-50	-	15
Thawing, ramp	-50	+25	1	75
Thawing	+25	+25	-	15
Freezing, ramp	+25	-50	1	75
Freezing	-50	-50	-	15
Thawing, ramp	-50	+25	1	75
Thawing	+25	+25	-	15
Freezing, ramp	+25	-50	1	75
Freezing	-50	-50	-	15
Thawing, ramp	-50	+25	1	75
Thawing	+25	+25	-	15

Light stress

Vials were treated in the sun tester at 750 W/m^2 and 25 °C for 7.5 hours in upside-down position.

Agitation

Vials were treated on a shaker at 200 rpm and 25 °C for 5 days in upside-down position.

Thermal stress

Vials were stored at 40 °C for 5 days in upside-down position.

3.2 Analytical methods

3.2.1 Absorption measurements

Samples were analyzed using a Cary 60 UV-spectrometer (Agilent Technologies, Santa Clara, CA, USA) by absorption measurement at 280 nm for concentration determination as well as at 350 nm and 510 nm for the evaluation of potential turbidity caused by the stress treatment. Samples were measured at a concentration of about 1 mg/mL (dilution with formulation buffer) or undiluted if the concentration was below 1 mg/mL using plastic cuvettes with an optical path length of 1.0 cm. The concentration was calculated according to Lambert-Beer´s law using an extinction coefficient of 0.974 mL/(mg*cm) as provided by Wacker Biotech for GM-CSF.

3.2.2 SE-HPLC (size exclusion chromatography) with Acquity BEH SEC

Chromatographic parameters

Mobile phase: 4.5 mM Na_2HPO_4, 77.6 mM NaCl, 10 mM KH_2PO_4, pH 7.4

Flow rate: 0.3 mL/min

Temperature: 28 ± 2 °C

UV-detection: 214 nm

Injection volume: 6 µL at 0.3 mg/mL GM-CSF (1.8 µg)

Sample temperature: 4 ± 2 °C

Buffer and sample preparation:

Table 10: Buffer and sample preparation

Buffer/solution	Composition/Description	Preparation procedure
Mobile phase	4.5 mM di-sodium hydrogen phosphate, 77.6 mM sodium chloride, 10 mM potassium dihydrogen phosphate, pH 7.4	0.64 g of di-sodium hydrogen phosphate, 4.53 g of sodium chloride, and 1.36 g of potassium dihydrogen phosphate were dissolved in 950 mL of purified water before the pH was
Reference solution	1 mg/mL GM-CSF in mobile phase	GM-CSF Control Sample 1 was diluted to 1.0 mg/mL with mobile phase.
Sample solution	Stressed samples	Stressed samples were measured undiluted at the target GM-CSF concentration of about 0.3 mg/mL.

3.2.3 SE-HPLC (size exclusion chromatography) with Superdex S200

Due to massive performance issues with the SE-HPLC method described in section 3.2.2 above, another method was implemented and used for the analysis of stressed formulation variants.

Chromatographic parameters

Mobile phase: 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0

Flow rate: 0.5 mL/min

Temperature: ambient

UV-detection: 214 nm and 280 nm

Injection volume: 100 µL at 0.3 mg/mL GM-CSF

Sample temperature: 4 ± 2 °C

Table 11: Buffer and sample preparation

Buffer/solution	Composition/Description	Preparation procedure
Mobile phase	0.1M sodium phosphate, 0.1M sodium chloride, pH 7.0	11.53 g of 85 % (w/w) phosphoric acid and 5.84 g of sodium chloride were topped with 900 mL with purified water in a 1000 mL glass beaker. The pH was adjusted to 7.0 with sodium hydroxide before the volume was filled up to 1000 mL with purified water.
Reference solution	0.3 mg/mL GM-CSF in mobile phase	GM-CSF Control Sample 1 was diluted to 0.3 mg/mL with mobile phase.
Sample solution	Stressed samples	Stressed samples were measured undiluted at the target GM-CSF concentration of about 0.3 mg/mL.

3.2.4 IEX-HPLC (ion exchange chromatography)

Chromatographic parameters

Mobile phase A: 20 mM KH_2PO_4 / KOH; pH 6.8

Mobile phase B: 20 mM KH_2PO_4 / KOH + 360 mM NaCl; pH 6.8

Flow rate: 1.0 mL/min

Temperature: 28 ± 2 °C

UV-detection: 214 nm

Injection volume: 2 µL at 1 mg/mL (2 µg)

Sample temperature: 4 ± 2 °C

Gradient

Table 12 - Gradient

Time [min]	Pump B conc. [%]
0.01	10
7.75	10
37.75	100
42.00	100
42.75	100
43.65	10
52.90	10
60.00	Stop

Table 13 - Buffer and sample preparation.

Buffer/solution	Composition/Description	Preparation procedure
Mobile phase A	20 mM KH_2PO_4 / KOH, pH 6.8	2.72 g of potassium dihydrogen phosphate were diluted in 900mL of purified water before the pH was adjusted to 6.8 using potassium hydroxide. Subsequently the volume was filled up to 1000mL using purified water.
Mobile phase B	20 mM KH_2PO_4 / KOH + 360 mM NaCl, pH 6.8	2.72 g of potassium dihydrogen phosphate and 21.04 g of sodium chloride were diluted in 900 mL of purified water before the pH was adjusted to 6.8 using potassium hydroxide. Subsequently the volume was filled up to 1000 mL using purified water.
Reference solution	1.0 mg/mL GM-CSF in mobile phase A	GM-CSF Control Sample 1 was diluted to 1.0 mg/mL with mobile phase A.
Sample solution	Stressed samples	Stressed samples were measured undiluted at the target GM-CSF concentration of about 0.3 mg/mL.

3.2.5 RP-HPLC (reversed-phase chromatography)

Chromatographic parameters

Mobile phase A: 0.1 % TFA in purified water

Mobile phase B: 10 % purified water + 0.1 % TFA in ACN

Flow rate: 1.2 mL/min

Temperature: 50 ± 2 °C

UV-detection: 214 nm

Injection volume: 100 µL at 0.5 mg/mL

Sample temperature: 4 ± 2 °C

Gradient

Table 14 - Gradient

Time [min]	Pump B conc. [%]
0.10	36
30	56

35	100
45	100
50	36
60	36

Table 15 - Buffer and sample preparation.

Buffer/solution	Composition/Description	Preparation procedure
Mobile Phase A	0.1 % TFA in purified water	1 mL of trifluoroacetic acid was mixed with 1000 mL of purified water.
Mobile Phase B	10 % purified water + 0.1 % TFA in ACN	1 mL of trifluoroacetic acid was mixed with 100 mL of purified water and 900 mL of acetonitrile.
Dilution buffer	0.05 M phosphate buffer, pH 7.0	2.63 g of potassium dihydrogen phosphate and 4.33 g of disodium hydrogen phosphate were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.0 using sodium hydroxide and the volume was filled up to 1000 mL with purified water.
Reference solution	0.5 mg/mL GM-CSF in dilution buffer	GM-CSF Control Sample 1 was diluted to 0.5 mg/mL with dilution buffer.
BSA solution	0.125 mg/mL BSA in dilution buffer	1.25 mg of BSA was diluted in 10 mL of dilution buffer.
SST solution	0.1 mg/mL GM-CSF and 0.1 mg/mL BSA in dilution buffer	100 µL of reference solution and 400 µL of BSA solution were mixed.
Sample solution	Stressed samples	Stressed samples were measured undiluted at the target GM-CSF concentration of about 0.3 mg/mL.

3.2.6 SDS-PAGE

Electrophoresis parameters

The electrophoresis parameters are shown in Table 16.

Table 16 – Electrophoresis parameters.

Conditions	Time [min]	Voltage [V]	Current [mA]	Output [W]
non-reducing	45	200	120	25

reducing	40	200	120	25

Buffer and sample preparation

Table 17 - Buffer and sample preparation.

Buffer/solution	**Composition/Description**	**Preparation procedure**	
Running buffer	1x MES electrophoresis buffer	150 mL of 20x MES electrophoresis buffer were filled up to a total volume of 3000 mL using purified water.	
Dilution buffer	15 mM Tris/HCL, pH 7.3	0.908 g of Tris were diluted in 495 mL of purified water before the pH was adjusted to 7.3 with 1N HCL. Subsequently the volume was filled up to 500 mL with purified water and the solution was filtered through a 0.22 µm PES filter.	
Reference pre-dilution	0.07 mg/mL GM-CSF + 0.07 mg/mL rHA in dilution buffer	GM-CSF Control Sample 1 as well as Recombumin® Alpha (rHA) were diluted to 0.14 mg/mL with dilution buffer before equal volumes of both solutions were mixed.	
Sample pre-dilution	0.07 mg/mL GM-CSF in dilution buffer	Samples were diluted to a GM-CSF concentration of 0.07 mg/mL using dilution	
Sample/reference solution	Sample or reference solution for loading onto the gel	Volume	Solution
		32.5 µL	Sample/reference pre-dilution
		12.5 µL	NuPAGE LDS (Sample Buffer
		5 µL	Purified water (non-reducing)
		or 5 µL	DTT (reducing)
		50 µL	Total volume

Table 18 - Gel loading pattern.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Analyte	Marker	T0 air	T0 N2	Fr/Th air	Fr/Th N2	Light air	Light N2	Shake air	Shake N2	40 °C	40 °C N2	Ref
Volume [µL]	5	10	10	10	10	10	10	10	10	10	10	10

4. Results and discussion

The following sub-variants (Table 19) were evaluated in the stress study, based on test variants as listed in Table 6.

Table 19 - Sub-variants evaluated in the forced degradation study. GM-CSF concentration was 0.3mg/mL for all variants.

Sub-variant #	Formulation	Composition	Overlay
1 air	10 mM K-phosphate, 260 mM sucrose, pH 7.3	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL	O_2
1 (PS20) air	10 mM K-phosphate, 260 mM sucrose, pH 7.3, 0.1% PS20	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL PS20: 1 mg/mL	O_2
1 (P188) air	10 mM K-phosphate, 260 mM sucrose, pH 7.3, 0.1% P188	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL P188: 1 mg/mL	O_2
1 (rHA) air	10 mM K-phosphate, 260 mM sucrose, pH 7.3, 0.1% rHA	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL rHA: 1 mg/mL	O_2
1 N2	10 mM K-phosphate, 260 mM sucrose, pH 7.3	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL	N_2
1 (PS20) N2	10 mM K-phosphate, 260 mM sucrose, pH 7.3, 0.1% PS20	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL PS20: 1 mg/mL	N_2
1 (P188) N2	10 mM K-phosphate, 260 mM sucrose, pH 7.3, 0.1% P188	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL P188: 1 mg/mL	N_2
1 (rHA) N2	10 mM K-phosphate, 260 mM sucrose, pH 7.3, 0.1% rHA	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL rHA: 1 mg/mL	N_2
2 air	10 mM K-phosphate, 260 mM Arginine/H_3PO_4, pH 7.3	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL	O_2

2 (PS20) air	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 7.3, 0.1% PS20	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL PS20: 1 mg/mL	O_2
2 (P188) air	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 7.3, 0.1% P188	Phosphoric acid: 0.98mg/mL L-arginine: 45.3 mg/mL P188: 1 mg/mL	O_2
2 (rHA) air	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 7.3, 0.1% rHA	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL rHA: 1 mg/mL	O_2
2 N2	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 7.3	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL	N_2
2 (PS20) N2	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 7.3, 0.1% PS20	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL PS20: 1 mg/mL	N_2
2 (P188) N2	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 7.3, 0.1% P188	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL P188: 1 mg/mL	N_2
2 (rHA) N2	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 7.3, 0.1% rHA	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL rHA: 1 mg/mL	N_2
3 air	10 mM K-phosphate, 260 mM sucrose, pH 8.1	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL	O_2
3 (PS20) air	10 mM K-phosphate, 260 mM sucrose, pH 8.1, 0. % PS20	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL PS20: 1 mg/mL	O_2
3 (P188) air	10 mM K-phosphate, 260 mM sucrose, pH 8.1, 0.1% P188	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL P188: 1 mg/mL	O_2
3 (rHA) air	10 mM K-phosphate, 260 mM sucrose, pH 8.1, 0.1% rHA	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL rHA: 1 mg/mL	O_2
3 N2	10 mM K-phosphate, 260 mM sucrose, pH 8.1	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL	N_2

3 (PS20) N2	10 mM K-phosphate, 260 mM sucrose, pH 8.1, 0.1% PS20	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL PS20: 1 mg/mL	N_2
3 (P188) N2	10 mM K-phosphate, 260 mM sucrose, pH 8.1, 0.1% P188	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL P188: 1 mg/mL	N_2
3 (rHA) N2	10 mM K-phosphate, 260 mM sucrose, pH 8.1, 0.1% rHA	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL rHA: 1 mg/mL	N_2
4 air	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 8.1	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL	O_2
4 (PS20) air	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 8.1, 0.1% PS20	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL PS20: 1 mg/mL	O_2
4 (P188) air	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 8.1, 0.1% P188	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL P188: 1 mg/mL	O_2
4 (rHA) air	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 8.1, 0.1% rHA	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL rHA: 1 mg/mL	O_2
4 N2	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 8.1	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL	N_2
4 (PS20) N2	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 8.1, 0.1% PS20	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL PS20: 1 mg/mL	N_2
4 (P188) N2	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 8.1, 0.1% P188	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL P188: 1 mg/mL	N_2
4 (rHA) N2	10 mM K-phosphate, 260 mM Arginine/ H_3PO_4, pH 8.1, 0.1% rHA	Phosphoric acid: 0.98 mg/mL L-arginine: 45.3 mg/mL rHA: 1 mg/mL	N_2

CF (330 µL fill after reconstitution)	0.3 mg/mL GM-CSF, human serum albumin (HSA), mannitol, macrogol 4000, dibasic sodium phosphate and monobasic potassium phosphate (all in unknown concentrations)		O_2

Sub-variants were exposed to the stress conditions as summarized in the set-up in Table 20 below.

Table 20 - Forced degradation sampling points for all sub-variants with (N_2) and without (O_2) degassing

0.3 mg/mL, 0.7 mL fill, per sub-variant (16 variants w/o degassing plus CF, 16 variants w/ degassing plus CF)				
	T0	**T7.5 hours**	**After freeze-**	**T5 days**
10 °C below T_{onset}	2 vials (+2 spare vials) 8 vials CF			2 vials (+2 spare vials) 8 vials CF
Agitation				2 vials (+2 spare vials) 8 vials CF
Exposure to light		2 vials (+2 spare vials) 8 vials CF		
Freeze-thaw			2 vials (+2 spare vials) 8 vials CF	
sum	4 vials	4 vials	4 vials	8 vials
Total for samples w/o degassing				20 vials per variant; 40 vials of current formulation
Total for samples w/ degassing and nitrogen overlay				20 vials per variant; 40 vials of current formulation

After exposing to stress and prior analysis, samples were visually inspected for turbidity/precipitation. No visible sign of particles causing turbidity or visible precipitates was identified in any of the samples. As an example, a picture of one of the samples that showed the weakest performance in the forced degradation study is shown in Figure 5 (sub-variant #2, with PS20, with N_2, light stress). No visible sign of turbidity or precipitation was observed.

4.1 Concentration and turbidity by absorption measurements

Absorption was measured at 280 nm in order to evaluate the GM-CSF concentration after stress application as well as at 350 nm and 510 nm to detect any increase in turbidity. The determined concentrations are listed in Table 21.

Table 21 - GM-CSF concentrations in mg/mL in the stressed samples by adsorption measurement at 280 nm.

		T=0		Fr/Th		Light		Shake		40 °C	
		Air	N_2	Air	N_2	Air	N_2	Air	N_2	Air	N_2
C	Control *										
#1	w/o	0.29	0.31	0.31	0.29	0.27	0.28	0.34	0.33	0.34	0.34
	PS20	0.33	0.33	0.31	0.31	0.30	0.31	0.35	0.37	0.35	0.35
	P188	0.31	0.33	0.31	0.30	0.29	0.29	0.35	0.35	0.34	0.35
	rHA*										
#2	w/o	0.33	0.40	0.38	0.35	0.41	0.32	0.36	0.35	0.37	0.34
	PS20	0.35	0.36	0.39	0.38	0.34	0.43	0.35	0.34	0.41	0.41
	P188	0.36	0.35	0.32	0.38	0.34	0.39	0.37	0.38	0.37	0.34
	rHA*										
#3	w/o	0.33	0.35	0.34	0.33	0.32	0.35	0.33	0.33	0.32	0.33
	PS20	0.35	0.35	0.36	0.34	0.33	0.35	0.34	0.34	0.33	0.35
	P188	0.35	0.34	0.34	0.34	0.32	0.33	0.32	0.32	0.32	0.32
	rHA*										
#4	w/o	0.30	0.31	0.30	0.30	0.30	0.30	0.29	0.30	0.31	0.31
	PS20	0.32	0.33	0.32	0.32	0.32	0.33	0.32	0.33	0.33	0.34
	P188	0.31	0.32	0.31	0.31	0.31	0.32	0.30	0.31	0.32	0.32
	rHA*										

* non-interpretable due to absorption measurement results at 280 nm being a combination of GM-CSF with HSA (in “control”) and with rHA in stressed samples, respectively.

The concentrations determined by UV absorption measurement at 280 nm showed rather high fluctuations (see e.g. sub-variant #2 PS20 at 40 °C). This was most probably caused by evaporation of water in the small sample volume leading to a concentration of the solutes in the small sample volume of roughly ~200 µL used for absorption measurements (repeat

measurement was not possible as remainder 500 µL were used for other analytical evaluation). However, the concentrations never significantly dropped below the target GM-CSF concentration of 0.3 mg/mL.

The measurements at 350 nm and 510 nm did not show any non-acceptable increase of turbidity (> 0.1 AU). Merely in the following sub-variants of test variant #2 a somewhat elevated turbidity could be detected at 350 nm:

w/o N_2 (T=0): 0.052 AU

w/o air (light): 0.054 AU

PS20 N_2 (light): 0.065 AU

rHA air (shake): 0.073 AU

The fact that absorption values higher than 0.05 occurred only in sub-variants derived from test variant #2 is a first weak indicator for the inferiority of this formulation compared to the others.

4.2 Aggregate status

The aggregate status of stressed samples was evaluated by SE-HPLC and SDS-PAGE, respectively.

4.2.1 SE-HPLC

"Control":

Due to performance issues the evaluation of the stressed "control" samples was done with a different SE-HPLC method (see section 3.2.2) than the formulation variants (see section 3.2.3).

The content of dimeric and even higher order aggregates of human serum albumin (HSA) is much higher in the "control" than in the sub-variant samples with rHA. In fact, at T=0 the aggregate peak of human serum albumin makes up for about 6.5 % of the total peak area, whereas in the sub-variants with rHA the dimer content was only at about 1.0 %. See Figure 6 for a representative chromatogram of the "control".

Other than that, the "control" did not show anything unexpected. HSA masks potential GM-CSF aggregates and, thus, the SE-HPLC method was not a good tool for the aggregation status evaluation of GM-CSF in GM-CSF current formulation HSA-containing samples (existing commercial formulation; "current DP"). Interestingly, the aggregate content decreased by applying stress (Figure 8) which might be due to precipitation.

Test variants:

The chromatograms obtained with the SE-HPLC method (section 3.2.3) were overall well assessable. Merely the chromatograms of the samples containing rHA could not be considered

exact, as the rHA peak (RT at ~28 min) and the main aggregation peaks of GM-CSF (~22 min - 31 min) were not separated by the method. As for the "control" it must be assumed that at least some of the aggregate content of GM-CSF is masked by rHA peaks and therefore gets underrated. The representative chromatograms displayed in Figure 7 elucidate the issue.

In general, all detected peaks that could not be identified as either monomeric GM-CSF or monomeric rHA were considered aggregates/impurities. For the determination of the relative GM-CSF content only the GM-CSF peak and all impurity peaks were integrated, but not the rHA monomeric peaks. Hence, aggregates deriving from the rHA (e.g. rHA dimers, RT 22.8 min) are displayed as impurities of GM-CSF as well.

The graph displayed in Figure 8 shows the minimum relative GM-CSF peak areas (without integration of the rHA monomeric peaks) of all stress conditions throughout the entire forced degradation study, to compress and visualize the huge amount of data. The sub-variants without additive (w/o) and the sub-variants containing poloxamer 188 (P188) showed the best results overall. Due to a high dimer content and the described masking effect of the HSA/rHA main peak, the results of the "control" as well as of the rHA-containing variants are not comparable with the sub-variants without HSA/rHA. The values for rHA and "control" in Figure 8 are only shown for completeness of the data.

The monomer content in the sub-variants without additive and with P188 only drops below 99% in sub-variant #2 (without additive with air, as well as with P188 without air) and once without additive in sub-variant #3 (without air). The sub-variants containing polysorbate 20 (PS20) and rHA, respectively, show monomer contents of less than 99% in each scenario for at least one stress condition and, hence, are considered inferior to sub-variants without additive and with P188.

4.2.2 SDS-PAGE

"Control" and test variants:

The aggregate status of stressed GM-CSF samples was further evaluated by SDS-PAGE. None of the gels showed any aggregate bands, which indicates rather low aggregate contents. Merely in the rHA-containing sub-variants very light/barely visible bands at around 150 kDa can be seen. These bands probably represent the rHA dimer fraction that was also observed by SE-HPLC.

The “control” however showed well visible aggregate bands in the >150 kDa area confirming again the SE-HPLC results. The samples contain aggregates that are most probably dimeric and multimeric human serum albumin (HSA) molecules in a much higher concentration than in the stressed test variants.

The protein bands were slightly more retarded in the reduced samples which accounts for disulfide bonds being reduced by the DTT, thus enlarging the molecule by unfolding.

4.3 Chemical degradation

Chemical degradation/fragmentation was evaluated by RP-HPLC as well as by IEX-HPLC.

4.3.2 RP-HPLC

“Control” and formulation variants:

Similar to the SE-HPLC, the obtained chromatograms were well assessable, whereby rHA also tends to mask some of the impurities thus letting the rHA-containing sub-variants seem purer than they actually are. See the chromatograms in Figure 11 for a representative example.

The evaluation of the chromatograms was performed exactly as described for SE-HPLC: The relative GM-CSF purities only take the GM-CSF peak as well as all impurity peaks into account, whereby the rHA monomeric peaks were not integrated at all for better comparability between the variants.

Again, the sub-variants w/o additive and, respectively, with P188 showed the highest purities overall. Furthermore, test variant #3 (10 mM potassium phosphate + 260 mM sucrose, pH 8.1, with and without air) showed the best results overall, whereas test variant #2 (10 mM potassium phosphate + 260 mM L-arginine, pH 7.3, with and without air) showed the lowest purities.

Annotation: the respective rHA-containing sub-variant does in fact show a similar purity as P188 in test variants #1 and #3, but this sub-variant is actually not comparable to the other sub-variants as impurities of GM-CSF get masked by rHA (as described in section 4.2.1 for the SE-HPLC results, and also clearly visible in Figure 11 with regard to RP-HPLC analysis). If the detected purity of the rHA sub-variants had been much higher than for the others, only then it would have been necessary to investigate rHA-containing sub-variants further. But since sub-variants w/o additive as well as with P188 are mostly at about the same purity, rHA-containing sub-variants should be neglected keeping in mind the masked impurities that cannot be seen in the graph.

Despite the low purities at pH 7.3, the L-arginine sub-variants at pH 8.1 (#4) show acceptable results which accounts for a high pH sensitivity of GM-CSF in this buffer. However, out of safety aspects, the results of test root-variant #4 (10 mM potassium phosphate + 260 mM L-arginine, pH 8.1, with and without air) should be treated with caution. Taking into account this high pH sensitivity in the presence of L-arginine and a compounding tolerance at the CMO of probably around 0.2 pH units (plus/minus), the very good results of test root-variant #4 become somewhat overshadowed as a slight difference in pH may significantly negatively affect the product's stability.

The analysis by RP-HPLC revealed the inferiority of the "control" formulation compared to test variants #1 and #3 (see Figure 12). Merely in test variant #4 was the minimum purity in the rHA-containing sub-variant lower than in the "control". The minimum detected purities cumulative throughout all stress conditions are shown in Figure 12.

5. Conclusions

Throughout the GM-CSF forced degradation study four test root-variants with 0.3 mg/mL GM-CSF and four different additives were tested in the presence and, respectively, in the absence of oxygen resulting in a total of 32 sub-variants. Additionally, a reconstituted GM-CSF lyophilizate containing HSA as a stabilizer underwent the same treatment to serve as a reference ("control"). The tested additives in the sub-variants were polysorbate 20 (PS20), poloxamer 188 (P188), rHA, and water as a baseline. All samples were stressed by light, shaking, elevated temperature at 40 °C, and multiple freeze/thaw cycles in order to discriminate the different formulation variants with respect to the stability of GM-CSF.

Several methods were applied for the evaluation of the purity and aggregate status of the drug product:

Absorption measurements at 280 nm, 350 nm, and 510 nm:

- A high fluctuation in concentration was detected which was probably caused by evaporation of water from the small sample volume of 200 µL.
- Measurements at 350 nm and 510 nm did not reveal any significant turbidity. Merely some sub-variants of test root-variant #2 did show absorption > 0.05 AU indicating the inferiority of this formulation compared to the others.

Aggregate status by SDS-PAGE:

- No aggregates could be detected in the sub-variants. Merely the rHA dimer was slightly visible.

- The "control" showed much thicker aggregate bands in the HSA dimer/multimer range accounting for a higher content of albumin compared to the sub-variants.

Aggregate status by SE-HPLC:

- The results are in line with the SDS-PAGE as, in the sub- variants, only small aggregate content could be detected but rather large amounts of HSA aggregates in the "control" - >6.5% HSA aggregates in the "control" compared to roughly 1% rHA aggregates in the sub- variants.
- Aggregate status evaluation of rHA- and HSA-containing samples is not very exact as the rHA monomer peak and HSA monomer peak masks potential GM-CSF aggregate peaks.
- PS20-containing sub-variants yielded the worst results, P188-ontaining sub- variants and sub-variants w/o additive, respectively, yielded the best results.
- No significant difference could be detected between sub-variants w/ and w/o air atmosphere. No oxidation events could be detected.
- Test variant #2 seems to be the weakest formulation amongst the four formulations, test variant #3 the best candidate formulation. Test variant #1 seems to be quite comparable to test variant#3 and, thus, test variant #1 would be preferred on second place. As there seems to be a quite distinct pH sensitivity of the test composition as in test variants #2 and #4, test variant #4 would also not be considered one of the preferred candidates as it would "only" be different from test variant #2 in pH and this pH sensitivity/dependence weakens the performance of test variant #4, as it could bear some risks with regard to product stability.

Chemical degradation by RP-HPLC:

- The RP-HPLC yielded the most discriminating results. Some formulations lost more than 50 % of their purity.
- The relative main peak area of rHA- and HSA-containing sub-variants again benefits from the rHA/HSA monomer peak as it masks GM-CSF degradation products.
- Test variant #2 yielded extremely bad results and should be dismissed completely from any further tests.
- Test variants #1, #3, and #4 all yielded good results, whereby #3 was superior over the others.
- Sub-variants with P188 as well as w/o additive seem to be the most stable. Sub-variants with PS20 showed the worst performance.
- The "control" was slightly inferior to the best variants.

Summarizing the results, test variants #1, #3, and #4 containing P188 and no additive were champions of this investigation. Test variant #4, however, is the same formulation as the very unfavorable test variant #2 but at a higher pH (8.1 compared to 7.3) which might lead to complications during production where a compounding tolerance of about 0.2 pH units usually has to be granted. This tolerance in combination with the observed high pH sensitivity poses a risk to the GM-CSF stability. rHA-containing variants were not bad overall but were potentially overrated due to the masking effect of the rHA peak in chromatograms. Furthermore, the use of rHA as an additive is rather inconvenient regulatory-wise as well as supply-wise.

At the bottom line, test variants #1 and #3 containing P188 and no additive, respectively, yielded the overall best results and should be looked further into for formulation development.

Example 3

1. Scope and Objective

The objective of this development work was to evaluate the presence of nanoparticles by dynamic light scattering (DLS) measurements in selected sub-variants from the forced degradation study of Example 2. A DynaPro Plate Reader (Wyatt Technology) was to be used for DLS measurements of nanoparticles in a microwell plate by measurement of the hydrodynamic radius (applicable range: from 0.1 nm to 1000 nm).

From each of those samples, two spare vials were available per time point and stress condition. While one spare vial of 4 selected candidates has already been used for external potency testing, the second remainder spare vial of those 4 samples was to be used to evaluate the presence of nanoparticles by DLS. Tested variants are listed in Table 22.

Table 22 – Sub- variants to be tested for nanoparticles by DLS.

Sample #	Sub-Variant #	Description
1	1(P188) air	0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 7.3; air overlay, T=0
2	1 (P188) air	0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 7.3; air overlay, T=5 d (at 40 °C)
3	3 (P188) air	0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 8.1; air overlay, T=0
4	3 (P188) air	0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 8.1; air overlay, T=5 d (at 40 °C)
5 (ref)		GM-CSF Control Sample 2: 5 mg/mL GM-CSF, 20 mM K-phosphate, pH 7.2

2. Materials and Apparatus

2.1 Substances and Materials

Table 23 lists substances and materials used during nanoparticle analysis.

Table 23 - Materials and substances used during nanoparticle analysis by DLS.

Substances	**Lot no.:**	**Manuf. / Suppl.**	**Order no.:**
GM-CSF Control Sample 2	160126/1089/082	-	-
Sub-variant #1 (P188) air	-	PJP (Example 2)	-
Sub-variant #3	-	PJP (Example 2)	-
Materials			
394-well plates for DLS	506301708017	Aurora	ABA200000A

*5 mg/mL GM-CSF, 20 mM K-phosphate, pH 7.2 (used as reference sample #5 in this study)

2.2 Apparatus

The following apparatus and equipment was used:

DLS plate reader

- Manufacturer: Wyatt Technology Corporation (Santa Barbara, CA, USA)
- Type: Dynapro plate reader II

Additional Laboratory Equipment

- Volumetric pipets (Gilson)

All equipment that was used undergoes regular calibration and qualification according to the valid qualification and maintenance plan.

3. Methods

3.1 Dynamic light scattering (DLS) measurement with DynaPro Plate reader

The principle of the Wyatt DynaPro Plate reader II is based on dynamic light scattering. When in solution a random motion of macromolecules, called Brownian motion, is observed, the movement of the molecules in a specific solution is detected as light intensity fluctuation over a defined time frame (e.g. 5 seconds). These fluctuations are directly related to the rate of diffusion of the molecule through the solvent, which is related in turn to the particles' hydrodynamic radius via the Stokes- Einstein equation:

$$D = \frac{kT}{6\pi\eta r}$$

D: diffusion coefficient

k: Boltzmann constant

T: absolute temperature

η: dynamic viscosity of the solvent

r: hydrodynamic radius

In general, smaller particles jitter around more rapidly, while larger particles diffuse more slowly. An overview of the measurement is displayed in Figure 13.

3.1.1 Sample preparation for DLS measurements

Samples were stored at -70 °C and were thawed at room temperature prior use. Each sample constituted a 2R vial with a fill volume of 0.7 mL and 0.3 mg/mL GM-CSF. Samples were filled into the wells of the measurement plate by portions of 30 µL and subsequently overlaid with silicone oil to avoid evaporation of liquid. No further treatment was applied before measurement.

4. Results and discussion

A DLS plate reader was used for determination of nanoparticles in selected samples from Example 2 (Table 22). Each sample was measured in triplicate, whereby the procedure was repeated once resulting in a total of six values per sample (each value was generated by 10 acquisitions). To simplify the complex set of data generated with the DLS plate reader, three ranges of particle sizes were defined (i.e. 0.1-10 nm, 10-100 nm, and 100-1000 nm) and the mass fraction of molecules covered by the specific range was calculated. Table 24 shows the mean radius measured in the specific range as well as the related mass fraction.

Table 24 - Results of the nanoparticle analysis by DLS.

Particle size range	Sample #	Mean particle radius [nm]	Mean mass fraction [%]
0.1 – 10 nm	1	1.51	99.7
	2	2.24	99.3
	3	2.10	99.8
	4	1.59	98.4
	5 (ref)	2.26	99.7
10 – 100 nm	1	38.1	0.18
	2	42.8	0.24
	3	37.6	0.03
	4	34.1	0.37
	5 (ref)	35.9	0.10
100 – 1000 nm	1	182	0.25
	2	301	0.53
	3	433	0.40

	4	506	0.82
	5 (ref)	385	0.03

In almost all samples as well as the reference the vast majority of particles by mass (i.e. > 99 % in average) was 0.1 - 10 nm large. Merely sample #4 (0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 8.1; air overlay, T=5 d at 40 °C) showed a somewhat higher content of larger particles (> 10 nm) of ~1.6 % in average. These small differences, however, should not be overrated considering the variance of the method.

With a molecular weight of 14.6 kDa, GM-CSF is a rather small molecule, where the mean particle radius would be expected in the measured range between 1 - 10 nm, which seems to correlate well with the high amount of mass fraction detected in this range.

As results for GM-CSF Control Sample 2 (sample #5), samples #1 and #3, and stressed samples #2 and #4 do all show the same cluster pattern within the detectable range of this method, it is not obvious that any treatment towards samples #1-#4 so far changed the nano-particle level of the GM-CSF Control Sample 2 in the range of 0.1 – 1,000 nm. Thus, samples #1 and #3 (sub-variants 1 and 3) seem to provide a stable matrix for GM-CSF with regard to nano-particle formation up to 1000 nm.

5. Conclusions

In the current study, samples corresponding to sub-variants from Example 2 (see Table 22) were analyzed with respect to nanoparticles via DLS measurement.

All samples, as well as the reference (GM-CSF Control Sample 2), showed comparable results, with more than 99 % (in average) of particles being 0.1 - 10 nm large (radius). Merely sample #4 (stressed sub-variant #3; 0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 8.1; air overlay, T=5 d at 40 °C) showed a somewhat higher content of larger particles (> 10 nm) of ~1.6 % in average, whereby this difference should not be considered significant.

As results for GM-CSF Control Sample 2 (sample #5), samples #1 and #3 and stressed samples #2 and #4 do all show the same cluster pattern within the detectable range of this method, it is not obvious that any treatment towards samples #1-#4 so far changed the nano-particle level of the BDS in the range of 0.1 - 1000 nm. Thus, samples #1 and #3 (sub-variants

#1 and #3) seem to provide a stable matrix for GM-CSF with regard to nano-particle formation up to 1000 nm.

Example 4

1. Scope and objectives

The objective of the overall development work was to develop a lyophilized formulation for GM-CSF drug product for intradermal application primarily in glass vial (30 µg in 100 µl injection volume, conc. ~0.3 mg/mL GM-CSF, fill volume ~330 µL (100 µl injection volume incl. 220 µl overfill) in standard 2R type I clear glass vial. Two final formulation variants determined from Example 2 sub-variant results were selected for subsequent development (Table 25, final formulation variants #1 and #2).

As for Fill & Finish a fill volume below 0.5 mL is a general issue, an increase of fill volume for lyophilization shall be evaluated in order to overcome any issue and to improve the filling accuracy. The final two formulations were diluted with 0.33 mL WFI to a volume of 0.66 mL prior lyophilization, for reconstitution in 0.33 mL WFI after lyophilization to achieve the targeted DP composition.

In this study, retrospectively predictive formulation analytics using CG-MALS for colloidal stability and nanoDSC for thermodynamic stability was to be performed on diluted final formulation variants (Table 25, #1d, #2d), like performed in formulation screenings in Example 1.

In order to evaluate a stabilization concept for GM-CSF in the diluted formulations, the developed stabilization concept for GM-CSF in the final formulation variants #1 and #2 (Table 25) was to be tested on the diluted final formulation variants:

- diluted final formulation variants were to be tested with respect to their feasibility for stabilizing the API
- the two final formulation variants served as reference
- preparation (by dialysis) of all four (4) final formulation variants (Table 25)
- analysis of all four (4) final formulation variants of the test matrix (measurement at ~ 5 mg/mL)
- determination of the colloidal stability (intermolecular interaction, A_2) by CG-MALS
- determination of the thermodynamic stability by nanoDSC.
- The four tested final formulation variants are listed in Table 25.

Table 25 – Final formulation variants tested in Example 4. d = diluted.

Final Formulation Variant #	Formulation
# 1	10 mM K-phosphate, 260 mM sucrose, (0.1 % (w/v) poloxamer 188)*, pH 7.3
# 2	10 mM K-phosphate, 260 mM sucrose, (0.1 % (w/v) poloxamer 188)*, pH 8.1
# 1d	5 mM K-phosphate, 130 mM sucrose, (0.05 % (w/v) poloxamer 188)*, pH 7.3
# 2d	5 mM K-phosphate, 130 mM sucrose, (0.05 % (w/v) poloxamer 188)*, pH 8.1

*Final formulation variants were prepared without poloxamer 188 (P188) in this example, as P188 is known to form micelles that might possibly interfere with the measurements as additional individual colloidal particles, thus possibly influencing the results for GM-CSF.

Based on the results, suitable formulations for Example 5 were to be selected.

2. Materials and apparatus

2.1 Substances and materials

Table 26 - Materials and substances

Substances	Lot no.:	Manuf. / Suppl.	Order no.:
GM-CSF Control Sample 2	160126/1089/082	-	-
ortho-phosphoric acid 85 %, EMPROVE® exp. Ph.Eur	K47168363542	Merck Chemicals	100563
Sodium chloride, EMPROVE® exp Ph. Eur., BP, USP	K93205800	Merck Chemicals	106400
Sucrose, EMPROVE® exp. Ph.Eur	K42784453205	Merck Chemicals	107653
Potassium chloride, ≥99 %, Ph.Eur., USP, BP	141168919	Carl Roth GmbH	P017
Sodium hydroxide solution, 30 % w/w pure, pharma grade	0000862568	AppliChem	144320.1211

Materials			
Slide-A-Lyzer dialysis cassettes, 15 mL; MWCO: 3.5 kDa	00600977	Thermo Scientific	87724
Minisart® PES 0.1 µm syringe filter	60395103	sartorius stedim	16553-K

*bulk drug substance for development (5 mg/mL GM-CSF, 20 mM K-phosphate, pH 7.2)

2.2 Apparatus

The following apparatus and equipment was used:

Purified water supply

- Manufacturer: Siemens
- Type: Ultra Clean UV UF TM

Static light scattering detector

- Manufacturer: Wyatt Technology Corporation (Santa Barbara, CA, USA)
- Type: Heleos

UV Spectrophotometer

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: Cary 60 UV-Vis
- SN: MY16170003

CG-MALS

- Manufacturer: Wyatt Technology Europe (Dernbach, Germany)
- Type: Calypso II
- Software: Calypso Version 2.1.3

nanoDSC

- Manufacturer: TA instruments (New Castle, DE, USA)
- Type: nanoDSC

Additional Laboratory Equipment

- Analytical balance (Kern)
- Magnetic stirrer (IKA Werke)
- pH meter (Mettler Toledo)
- Volumetric pipets (Gilson)

3. Methods

3.1 Buffer and sample preparation

3.1.1 Buffer preparation for dialysis

The preparation procedure for the four different final formulation variant buffers is described in Table 27.

Table 27 - Buffer preparation for the subsequent dialysis of GM-CSF bulk drug substance. d = diluted.

Solution	Composition	Preparation procedure
Final formulation variant #1	10 mM K-phosphate, 260 mM sucrose, pH 7.3	1.15 g of 85 % (w/w) ortho-phosphoric acid and 89.0 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.3 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
Final formulation variant #2	10 mM K-phosphate, 260 mM sucrose, pH 8.1	1.15 g of 85 % (w/w) ortho-phosphoric acid and 89.0 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
Final formulation variant #1d	5 mM K-phosphate, 130 mM sucrose, pH 7.3	0.575 g of 85 % (w/w) ortho-phosphoric acid and 44.5 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.3 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
Final formulation variant #2d	5 mM K-phosphate, 130 mM sucrose, pH 8.1	0.575 g of 85 % (w/w) ortho-phosphoric acid and 44.5 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.

3.1.2 Dialysis

The dialysis of GM-CSF was accomplished in three independent dialysis steps to achieve a quantitative buffer exchange. 8 mL of the GM-CSF drug substance (c = 3.5 mg/mL) was transferred into preconditioned (in dialysis buffer) Slide-A-Lyzer cassettes (Thermo Scientific, Rockford, USA). Filled Slide-A-Lyzer cassettes were incubated in 650 mL of the target buffer for 2 hours before a first buffer change (650 mL) was performed. After dialysis for two further hours

the buffer was changed a second time (650 mL) to finalize the dialysis overnight. The protein sample was removed from the Slide-A-Lyzer cassettes and processed further. Each dialysis step represents a 1/80 fold buffer exchange leading to a calculated buffer exchange factor of 5 x 10^5 in total.

3.2 UV measurement

The concentration of GM-CSF in solution was determined using a Cary 60 UV-spectrometer (Agilent Technologies, Santa Clara, CA, USA) by adsorption measurement at 280 nm. Samples were measured at a concentration of about 1 mg/mL using plastic cuvettes with an optical path thickness of 1.0 cm. The concentration was calculated according to Lambert-Beer´s law using an extinction coefficient of 0.974 mL/(mg*cm) as provided by Wacker Biotech for GM-CSF.

3.3 Nano Differential Scanning Calorimetry (nanoDSC)

Nano DSC is specifically designed to determine the denaturation temperature and thermal denaturation enthalpy of proteins and other macromolecules in solution with the versatility and precision to perform molecular stability screenings.

Experiment method:

Mode: Scanning

Temperature parameters:	Lower: 20 °C
	Upper: 100 °C
	Rate: 1 °C/min heating and cooling
	Equilibration: 600 s
Pressure parameters:	Manual, 3.0 atm
Data interval:	1 s

A buffer scan was conducted prior to each sample run to generate a baseline.

Sample preparation:

Samples were dialyzed against the corresponding formulation buffer (see section 3.1.2) and diluted to 1 mg/mL. The corresponding dialysis buffer was used as buffer scan and buffer reference.

Interpretation of analysis data:

Differential scanning calorimetry measures the amount of heat that is absorbed or released from biomolecules in solution during heating or cooling. Native proteins respond to heating by unfolding (thermal denaturation) at a characteristic temperature (T_m). The more intrinsically stable the biopolymer is the higher is the onset temperature of the unfolding transition and the transition enthalpy (H). The onset temperature of thermal denaturation, i.e. the starting point of the unfolding transition, is defined as T_{onset}.

3.4 Composition gradient multi-angle light scattering (CG-MALS)

Interactions between protein molecules in solution were characterized by changes in their light scattering behavior at different concentrations via MALS. With this series of light scattering measurements the second virial coefficient A_2, which is a characteristic parameter to evaluate molecule interactions, was calculated.

A_2 is characteristic for a substance and its solvent, and describes molecular interactions between the dissolved components. A negative A_2 indicates attractive interactions between molecules of the dissolved substance whereas a positive A_2 is characteristic for repulsive interactions between the dissolved protein molecules.

The CG-MALS technique is based on a series of differently concentrated macromolecular solutions which are directly injected into the flow cell of a multi-angle light scattering detector. After each injected concentration step the flow is stopped to permit the reaction to reach equilibrium for signal stabilization and subsequent detection. The apparent weight average molecular weight (Mw_{app}) is determined for each step in the concentration gradient by analyzing the light scattering and concentration data by the following equation:

$$Mw_{app} = \frac{R(\theta,c)}{K \times c}$$

Mw_{app}: apparent molecular weight

R(θ,c) **:** excess Rayleigh ratio of the solution as a function of scattering angle θ and concentration c. It is directly proportional to the intensity of the excess light scattered by the solute and the light scattered by the pure solvent.

K: Optical constant ($4p^2(dn/dc)^2n_0^2/N_al_0^4$)

c: Concentration

Significant interactions between macromolecules manifest as changes in Mw_{app} vs. concentration.

A_2 calculation was conducted via Zimm plot analysis by an extrapolation to 0 mg/mL concentration according to:

$$\frac{K^* c}{R(\theta,c)} = \frac{1}{M_w P(\theta)} + A_2 c$$

R(θ,c) **:** excess Rayleigh ratio of the solution as a function of scattering angle θ and concentration c. It is directly proportional to the intensity of the excess light scattered by the solute and the light scattered by the pure solvent.

M_w: Weight average molecular weight

A_2: 2nd virial coefficient

c: Concentration

K^*: Optical constant ($4p^2(dn/dc)^2n_0^2/N_al_0^4$)

P(θ): describes the angular dependence of the scattered light, and can be related to the rms radius

Sample preparation:

Samples were dialyzed against the corresponding final formulation variant buffer (see section 3.1.2) and used undiluted. The corresponding dialysis buffer was used as diluent for the CG-MALS experiment. The samples were passed over a 0.1 µm syringe filter. Before loading the sample into the CG-MALS system the exact concentration of the sample was determined by UV-absorption measurement (see section 3.2). Sample and buffer were loaded into the Calypso II system and the measurement was started.

Sample measurement:

A Calypso II CG-MALS system was used to supply the MALS detector with the concentration gradient of the analyte. The sample was loaded on one syringe pump of the system and the dialysis buffer on another syringe pump of the system. Ten equidistant concentration steps from approx. 3.5 mg/mL to 0.5 mg/mL were applied in each measurement by diluting the sample with dialysis buffer. At each gradient step 1 mL of sample was injected into the MALS detector. The resulted light scattering signal was recorded over a time period of 180 seconds. In addition to the light scattering signal the concentration of each gradient step was recorded using a refractive index detector. Control of the system and analysis of the data via Zimm plot analysis was performed with Calypso software version 2.1.5.

4. Results and Discussion

The most promising two selected formulation sub-variants for GM-CSF (Table 25), selected after the forced degradation study (Example 2), as well as the respective diluted versions (half of the original excipient concentration; Table 25) were evaluated in direct comparison to each other with respect to thermodynamic stability (by nanoDSC) and intermolecular interactions (by CG-MALS).

The tested final formulation variants as well as the respective results are listed in Table 28.

Table 28 - Results of the predictive formulation analytics II, of reference and diluted final formulation variants. d = diluted.

Final Formulation Variant #	Formulation	A_2 [mol x mL x g-2 x 10-4]	T_{onset} [°C]
# 1	10 mM K-phosphate, 260 mM sucrose, pH 7.3	-4.12	n.d.
# 2	10 mM K-phosphate, 260 mM sucrose, pH 8.1	1.48	n.d.
# 1d	5 mM K-phosphate, 130 mM sucrose, pH 7.3	2.66	n.d.
# 2d	5 mM K-phosphate, 130 mM sucrose, pH 8.1	13.4	n.d.

As in Example 1, final formulation variant #1 showed a negative A_2 indicating unfavorable attractive molecule-molecule interactions in solution, whereas the counterpart formulation with more basic pH (final formulation variant #2) showed a positive A_2 indicating repulsive molecule-molecule interactions in solution, lowering the tendency for aggregation events. In both cases the halving of the buffer molarity and of the excipient concentration (final formulation variants #1d and #2d; d = diluted) led to improved intermolecular interactions as A_2 values were both clearly positive. The rather high A_2 value for final formulation variant #2d should not be overrated as measurements were extremely noisy and thus hard to evaluate.

5. Conclusions

In order to evaluate a stabilization concept for GM-CSF in the diluted final formulation variants, targeting a fill volume for fill & finish prior lyophilization of > 0.5 mL, the developed stabilization concept for GM-CSF in the final two most promising formulations (see separate reports on Example 1 and Example 2) was tested on the diluted final formulation variants.

The results for colloidal stability (A_2 by CG-MALS) for the original final formulation variants #1 and #2 (Table 25) were in line with the results obtained in Example 1, i.e. the A_2 determined for final formulation variant #1 (pH 7.3) was negative indicating attractive molecule-molecule interactions in solution, whereas the A_2 in the more basic final formulation variant #2 (pH 8.1)

was positive indicating repulsive molecule-molecule interactions in solution. Halving the excipient concentration led to clearly positive A_2 values on both final formulation variants, accounting for a lower tendency towards aggregation and thus an improved stabilization of GM-CSF compared to the original final formulation variants.

In Example 1, onset of thermal denaturation was determined for final formulation variant #1 and #2 (corresponding to variants #rep. 13 and #19 in Table 4) to be at ~54 °C, a comfortable range far away from common handling and storage temperatures.

Example 5

1. Scope and objective

Two final formulation variants, as determined from Example 2, were selected for the feasibility of lyophilization. The final formulation variants were as in the Table 29 below.

Table 29 - Formulation variants (final formulation)

Final Formulation Variant #	**Formulation (final formulation)**	**Composition**	**Fill volume per vial**
1 (P188), air overlay **= #1**	0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, pH 7.3, 0.1 % P188	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL P188: 1 mg/mL	0.33 mL
2 (P188), air overlay **= #2**	0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, pH 8.1, 0.1 % P188	Phosphoric acid: 0.98 mg/mL Sucrose: 89.0 mg/mL P188: 1 mg/mL	0.33 mL

As for Fill & Finish a fill volume below 0.5 mL is a general issue, an increase of fill volume was evaluated in order to overcome any issue and to improve the filling accuracy. Final formulations variants were diluted with 0.33 mL WFI to a volume of 0.66 mL. The following “diluted” final formulation variants (Table 30) should be evaluated in parallel with the final formulation variants (Table 29).

Table 30 - Formulation variants (diluted formulation)

Final Formulation Variant #	**Formulation (diluted formulation; Estimated based on dilution factor)**	**Composition**	**Fill volume per vial**
1 (P188), air overlay, diluted **= #1d**	0.15 mg/mL GM-CSF, 5 mM K-phosphate, 130 mM sucrose, pH 7.3*), 0.05 % P188	0.33 mL final formulation variant (Table 29) + 0.33 mL WFI	0.66 mL
2 (P188), air overlay, diluted **= #2d**	0.15 mg/mL GM-CSF, 5 mM K-phosphate, 130 mM sucrose, pH 8.1*), 0.05 % P188	0.33 mL final formulation variant (Table 29) + 0.33 mL WFI	0.66 mL

*pH after dilution unknown

The four final formulation variants plus an additional placebo/vehicle formulation #2v (corresponds to #2 but without GM-CSF) were lyophilized in the pilot freeze dryer using a conservative lyophilization cycle (Table 34). After the lyophilization feasibility test run, the products were analyzed with respect to:

- visual appearance, documented by macro photography
- residual water content using a generic Karl-Fischer oven method (in duplicate)
- reconstitution behavior (using 0.33 mL WFI)
- pH measurement after reconstitution
- aggregation status by SE-HPLC
- degradation profile by RP-HPLC
- nano-particle content by DLS

For comparability purposes, an existing commercial formulation (w/ HSA, mannitol, macrogol 4000, dibasic sodium phosphate, monobasic potassium phosphate, “as is”; hereafter variant #C, lyophilized “control” formulation) was to be analyzed in parallel using the same methods.

In a first lyophilization trial the feasibility for freeze drying of previously selected formulation variants (see Table 31) was tested. The formulation variants were compounded. The preparations were terile filtered through 0.1 µm PES membrane filter and filled into cleaned and heat-sterilized 2R vials. Active vials were partially stoppered with autoclaved and dried lyo stoppers. Samples were freeze dried using a conservative prototype lyo-cycle.

An existing commercial formulation (w/ HSA, mannitol, macrogol 4000, dibasic sodium phosphate, monobasic potassium phosphate, “as is”) served as reference for analytics after lyophilization.

Table 31 - Formulation variants tested for lyophilization feasibility in Example 5

Final Formulation Variant #	**Composition of liquid bulk drug substance**	**Composition of lyophilized drug product per vial**	**Comment**
1	0.30 mg/mL, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 7.3	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 7.3)	Regular formulation #1 fill volume: 330 µL batch size: 160 vials
1d	0.15 mg/mL, 5 mM K-phosphate, 130 mM sucrose, 0.05 % (w/v) poloxamer 188, pH 7.3	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 7.3)	Diluted formulation #1 fill volume: 660 µL batch size:: 60 vials
2	0.30 mg/mL, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 8.1	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 8.1)	Regular formulation #2 fill volume: 330 µL batch size: 160 vials
2d	0.15 mg/mL, 5 mM K-phosphate, 130 mM sucrose, 0.05 % (w/v) poloxamer 188, pH 8.1	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 8.1)	Diluted formulation #2 fill volume: 660 µL batch size: 60 vials
2v	10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer	0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188	Vehicle/placebo formulation #2 fill volume: 330 µL

	188, pH 8.1	(potassium hydroxide to pH 8.1)	batch size: 40 vials

2. Materials and apparatus

2.1 Substances and materials

Table 32 – Substance and materials

Substances	**Lot no.:**	**Manuf. / Suppl.**	**Order no.:**
GM-CSF (Control Sample 1	150904/1064/71	-	-
GM-CSF Control Sample 3 *	170118/1167/095	-	-
Lyophilized ("control") formulation	A201501A02	-	-
Purified water (≤ 0.2 µmS/cm, < 10 ppb TOC)	-	PJP	-
Kolliphor® P 188, Poloxamer, Ph. EUR.	WPDH566B	BASF	50259527
ortho-phosphoric acid 85 %, EMPROVE® exp. Ph. Eur.	K47168363542	Merck Chemicals	100563
Sodium chloride, EMPROVE® exp Ph. Eur. BP, USP	K45515600	Merck Chemicals	106400
Sucrose, EMPROVE® exp. Ph.Eur	K42784453205	Merck Chemicals	107653
Sodium hydroxide solution, 30 % w/w pure, pharma grade	0000862568	AppliChem	144320.1211
Potassium hydroxide, EMPROVE® exp Ph Eur	B03389302	Merck Chemicals	105032
Potassium dihydrogen phosphate, ≥ 98 %, Ph.Eur., USP, BP	261168601	Carl Roth GmbH	P018
di-Sodium hydrogen phosphate, ≥ 98 %, Ph.Eur	373201802	Carl Roth GmbH	T876
Trifluoroacetic acid, ≥ 99.9 %, for peptide synthesis	245230492	Carl Roth GmbH	P088.1
Acetonitrile, Chromasolv gradient grade	SZBG041AV	Sigma-Aldrich	34851

Materials			
Slida-A-Lyzer dialysis cassettes, 15 mL; MWCO: 3.5 kDa	00601203	Thermo Scientific	87724
Minisart® PES 0.1 µm syringe filter	70681103	Sartorius stedim	16553-K
Jupiter 5 µm C18 (300 Å; 250 × 4.6 mm)	H16-035225	Phenomenex	00G-4053-E0
Superdex™ 200 Increase 10/300 GL	10246668	GE Healthcare	28-990o9-44
2R FIOLAX injection vials, clear	162263031	Nipro Germany GmbH	-
Lyo stopper, 1356 4023/50/GREY B2-TR Westar®RS SQS	1152012315	West	7002-3047
Crimp caps, aluminum, 13 mm, red	1103241615	Helvoet Pharma	STO13.0001003
Crimp caps, aluminum, 13 mm, blue	-	Bico Pharma	AG1302-1-005
Crimp caps, aluminum, 13 mm, gold	1302151315	Dätwyler Pharma Packaging	STO13.0001002
Crimp caps, aluminum, 13 mm, clear	1306241600	Dätwyler Pharma Packaging	STO13.0001001

* GM-CSF drug substance used for formulation of the final formulation variants #1, #2, #1d and #2d in this study (5 mg/mL GM-CSF, 20 mM K-phosphate, pH 7.2)

2.2 Apparatus

The following apparatus and equipment was used:

Purified water supply

- Manufacturer: Siemens
- Type: Ultra Clean UV UF TM

UV Spectrophotometer

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: Cary 60 UV-Vis
- SN: MY16170003

HPLC Equipment

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: 1100 Series
- Degasser G1322A
- BinPump G1312A
- ALS G1329A
- ALSTherm G1330A
- ColCom G1316A
- DAD G1315A

Pilot freeze dryer (GT1):

- Manufacturer: Hof Sonderanlagenbau (Lohra, Germany)
- 0.5 m^2 shelf area
- 10 kg ice condenser capacity
- Adjustable ice condenser temperature
- In-vial temperature recording
- Differential pressure measurement

Camera Equipment

- Manufacturer: Canon (Tokio, Japan)
- Body: EOS 550D/EOS 600D
- Lens: EFS 18-55mm, DGMacro 105mm 1:2.8

DLS plate reader

- Manufacturer: Wyatt Technology Corporation (Santa Barbara, CA, USA)
- Type: Dynapro plate reader II

Additional Laboratory Equipment

- Analytical balance (Kern)
- Magnetic stirrer (IKA Werke)
- pH meter (Mettler Toledo)
- Volumetric pipets (Gilson)
- Multistep Pipettes: Brand, HandyStep
- Autoclave: Systec, DX65
- Drying oven: Memmert, 500

- Orbital shaker: Wisd Laboratory Instruments, WiseShake SHO-1D
- Laminar flow microbiological class 2 work bench: BDK, UVF 6.18S

3. Methods

3.1 Preparation of liquid bulk drug substance

Sterilization of vials and stoppers:

2R glass vials were washed with purified water and subsequently depyrogenized and sterilized by dry heat at 300 °C for 2 hours.

Stoppers were autoclaved at 134 °C for 10 minutes and subsequently dried at 70 °C for 8 hours.

Preparation of formulation buffers:

Table 33 – Formulation buffers

Final Formulation variant #	Composition	Preparation procedure
1	10 mM K-phosphate, 260 mM sucrose, pH 7.3	1.15 g of 85 % (w/w) ortho-phosphoric acid and 89.0 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.3 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
2	10 mM K-phosphate, 260 mM sucrose, pH 8.1	1.15 g of 85 % (w/w) ortho-phosphoric acid and 89.0 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.

Dialysis:

The dialysis of GM-CSF drug substance was accomplished in three independent dialysis steps to achieve a quantitative buffer exchange. 6 mL of the GM-CSF drug substance (c = 5.0 mg/mL) were transferred into preconditioned (in dialysis buffer) Slide-A-Lyzer cassettes with a molecular weight cut off of 3.5 kDa and a maximum fill volume of 15 mL (Thermo Scientific, Rockford, USA). Filled Slide-A-Lyzer cassettes were incubated in 500 mL of the target buffer for 2 hours before a first buffer change (500 mL) was performed. After dialysis for two further hours the buffer was exchanged a second time (500 mL) to finalize the dialysis overnight. The protein sample was removed from the Slide-A-Lyzer cassettes and processed further. Each dialysis

step represented a 1/83 fold buffer exchange leading to a calculated buffer exchange factor of 1:5.7×10^5 in total. All dialysis steps were performed at 2-8 °C.

Compounding and Filling:

After dialysis the GM-CSF solution was analyzed via UV absorption measurement at 280 nm to determine the GM-CSF concentration. The solution was then diluted to a concentration of 0.3 mg/mL using formulation buffer before it was spiked with the required amount of poloxamer 188 to obtain a concentration of 0.1 % (w/v) detergent resulting in final formulation variants #1 and #2. In order to obtain the respective diluted final formulation variants #1d and #2d, portions of the readily compounded final formulation variants #1 and #2 were diluted with the same volume of water (1v+1v). Variant #2v (vehicle/placebo formulation) was formulation buffer #2 with 0.1 % (w/v) poloxamer 188 but without GM-CSF.

The readily compounded lyo solutions were sterile filtered using a 0.1 µm PES syringe filter and filled into sterile 2R glass vials by aliquots of 330 µL (final formulation variants #1, #2, and #2v) and 660 µL (final formulation variants #1d and #2d), respectively, using a multistep pipette. Subsequently the vials were partially stoppered with lyo stoppers in lyo position and placed in a freeze dryer. All compounding/filling steps were performed under a laminar flow bench to avoid microbial contamination of the product.

3.2 Lyophilization

The filled vials with lyo-stoppers attached to the vials in “lyo-position” (first snap of lyo stopper legs onto the vial, leaving lateral openings for flow of sublimation stream) were positioned on the shelves of the pilot freeze dryer. 160 vials were loaded per final formulation variant #1 and #2, 60 vials per final formulation variant #1d and #2d, and 40 vials of formulation variant #2v. The process parameters applied for lyophilization are listed in Table 34. A so called radiation cage was employed to actively cool the walls of the lyo chamber in order to simulate large scale conditions.

Table 34 - Lyophilization process parameters applied for lyophilization feasibility testing of GM-CSF DP

Step		**Shelf temperature**	**Pressure**	**Step time**	**Cumulative time**
#	Description	[°C]	[mbar]	[h:min]	[h:min]
1	Loading	20	atm	00:01	0:01
2	Freezing, ramp	-50	atm	01:10	1:11

3	Freezing	-50	atm	02:00	3:11
4	Vacuum Adjustment	-50	1,00E-01	00:30	3:41
5	Primary Drying, ramp	-10	1,00E-01	01:00	4:41
6	Primary Drying	-10	1,00E-01	44:00	48:41
7	Secondary Drying, ramp	35	1,00E-01	06:00	54:41
8	Secondary Drying	35	1,00E-01	15:00	69:41
9	Venting with N2/stopper closing	35	7,50E+02	00:10	69:51

To monitor the product temperature during freeze drying, thermocouples were inserted into product vials as displayed in Figure 14. Pressure was controlled during lyophilization by using a capacitive pressure sensor (MKS). Pressure regulation was managed via vacuum and dosing valve (nitrogen injection). After completion of secondary drying vials were closed at a pressure of 800 mbar under nitrogen atmosphere. Vials were unloaded from the freeze dryer and crimp capped (using a hand crimping tool).

3.3 Analytical methods

3.3.1 Absorption measurements

Samples were analyzed using a Cary 60 UV-spectrometer (Agilent Technologies, Santa Clara, CA, USA) by absorption measurement at 280 nm for concentration determination. Samples were measured at a concentration of about 1 mg/mL (dilution with formulation buffer) or undiluted if the concentration was below 1 mg/mL using plastic cuvettes with an optical path length of 1.0 cm. The concentration was calculated according to Lambert-Beer's law using an extinction coefficient of 0.974 mL/(mg*cm).

3.3.2 SE-HPLC (size exclusion chromatography)

Chromatographic parameters:

Mobile phase: 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0
Flow rate: 0.5 mL/min
Temperature: ambient
UV-detection: 214 nm and 280 nm
Injection volume: 100 µL at 0.3 mg/mL GM-CSF

Sample temperature: 4 ± 2 °C

Buffer and sample preparation:

Table 35 – Buffer and sample preparation.

Buffer/solution	Composition/Description	Preparation procedure
Mobile phase	0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0	11.53 g of 85 % (w/w) phosphoric acid and 5.84 g of sodium chloride were topped with 900 mL with purified water in a 1000 mL glass beaker. The pH was adjusted to 7.0 with sodium hydroxide before the volume was filled up to 1000 mL with purified water.
Reference solution	0.3 mg/mL GM-CSF in mobile phase	GM-CSF Control Sample 1 was diluted to 0.3 mg/mL with mobile phase.
Sample solution	Stressed samples	Stressed samples were measured undiluted at the target GM-CSF concentration.

3.3.3 RP-HPLC (reversed-phase chromatography)

Chromatographic parameters:

Mobile phase A: 0.1 % TFA in purified water

Mobile phase B: 10 % purified water + 0.1 % TFA in ACN

Flow rate: 1.2 mL/min

Temperature: 50 ± 2 °C

UV-detection: 214 nm

Injection volume: 100 µL at 0.5 mg/mL

Sample temperature: 4 ± 2 °C

Gradient:

Table 36 - Gradient

Time [min]	Pump B conc. [%]
0.10	36
30	56
35	100
45	100
50	36

60	36

Buffer and sample preparation:

Table 37 – Buffer and sample preparation.

Buffer/solution	Composition/Description	Preparation procedure
Mobile Phase A	0.1 % TFA in purified water	1 mL of trifluoroacetic acid was mixed with 1000 mL of purified water.
Mobile Phase B	10 % purified water + 0.1 % TFA in ACN	1 mL of trifluoroacetic acid was mixed with 100 mL of purified water and 900 mL of acetonitrile.
Dilution buffer	0.05 M phosphate buffer, pH 7.0	2.63 g of potassium dihydrogen phosphate and 4.33 g of disodium hydrogen phosphate were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.0 using sodium hydroxide and the volume was filled up to 1000 mL with purified water.
Reference solution	0.3 mg/mL GM-CSF in dilution buffer	GM-CSF Control Sample 1 was diluted to 0.3 mg/mL with dilution buffer.
Sample solution	Stressed samples	Stressed samples were measured undiluted at the target GM-CSF concentration.

3.3.4 Nano-particle analysis by DLS (dynamic light scattering)

The principle of the Wyatt DynaPro Plate reader II is based on dynamic light scattering, and is described in Example 3, section 3.1.

Sample preparation:

Each sample constituted a 2R vial with lyophilized GM-CSF drug product with a target GM-CSF amount of 0.10 mg per vial. After reconstitution with 0.33 mL WFI, samples were filled into the wells of the measurement plate by portions of 30 µL and subsequently overlaid with silicone oil to avoid evaporation of liquid. No further treatment was applied before measurement.

3.3.5 Process analytics of the lyophilization

The process data of the lyophilization (time, pressure and temperature readings including in-vial product temperature measurements), were logged by a controlling computer and visualized in a graph.

3.3.6 Visual appearance of the lyophilizate

Lyophilizate-containing vials were examined visually. Photographs were taken for documentation.

3.3.7 Karl-Fischer oven method

The residual moisture of the samples was determined via Karl Fischer titration using a 756 Karl Fischer coulometer equipped with a 774 oven sample processor (Metrohm).

For each measurement one lyophilizate of each sample (~20-30 mg of a lyophilizate) was transferred in a Karl-Fischer glass vial. The exact weight of the lyophilizate was documented. The vial was sealed with a crimp cap and transferred into the oven of the Karl Fischer coulometer which was heated to 100 °C. The septum of the crimp cap was penetrated by an injection needle, and the generated water vapour was directly transferred into the titration chamber of the Karl Fischer coulometer via dry nitrogen flow. Empty glass vials were used as blank correction. Samples were analyzed in duplicate.

3.3.8 Reconstitution

The vials were reconstituted by the addition of 330 µL WFI using a volumetric pipette after removing the cap and stopper from the vial. Subsequently the vial was gently shaken until complete dissolution of the lyo cake.

Specifically, 330µL WFI was added to the vial, and gently swirled for 2 minutes by hand, then allowed to stand at ambient temperature for 11 minutes. The lyophilized cake should reconstitute to a clear solution. Analysis can commence after the standing time – the length of time from reconstitution until start of analysis was noted in the test documentation.

4 Results and discussion

4.1 Lyophilization process online data

The chamber pressure was monitored by capacitive pressure sensor and Pirani pressure sensor and recorded via on-line data acquisition to detect the end of sublimation.

The lyophilization program was completed as planned. The recorded temperature and pressure profile complied with the predefined process parameters. The product temperatures measured via thermo couples indicated the end of the sublimation phase after about 9 hours total process time for final formulation variants #1 and #2, and after about 13 hours for final formulation variants #1d and #2d, respectively. The diluted final formulation variants had a longer sublimation phase due to the higher fill volume of the vials (0.66 mL as opposed to 0.33 mL). The entire cycle lasted 69 hours.

160 vials were freeze dried per final formulation variant #1 and #2, 60 vials per final formulation variant #1d and #2d, and 40 vials of formulation variant #2v.

4.2 Visual appearance of the lyo cakes

After lyophilization all cakes were examined visually with respect to shrinkage and cake defects. Overall, the obtained lyo cakes were in good, acceptable shape besides some mild shrinkage observed for all produced variants which is a common phenomenon with formulations mainly consisting of sucrose. The existing commercial formulation (#C, lyophilized “control” formulation), however, did not show shrinkage as expected for a mannitol cake. No major defects could be identified. Exemplary photographs of the lyophilizates are shown in Figure 15.

4.3 Residual water content

The residual water content in the lyophilizates was determined using a generic Karl-Fischer oven method (100 °C). The results are listed in Table 38.

Table 38 - Residual water content by Karl-Fischer oven method

Final Formulation Variant	Residual water content [%]
#1	0.3
#2	0.5
#1d	0.2
#2d	0.2
#C	0.6

All measured residual water values in the lyophilized samples were well below 1%. The current formulation showed a significantly higher content of water of 1.3%.

4.4 Reconstitution behavior

Reconstitution was performed by addition of 0.33 mL WFI and subsequent immediate and gentle shaking. The complete dissolution of the lyo cake of all final formulation variants #1, #2, #1d, #2d was achieved after about 10 seconds. The existing commercial formulation (#C) dissolved slightly slower than the newly developed variants.

4.5 pH after reconstitution

After reconstitution with 0.33 mL WFI the pH value of each variant was determined. The measured values are listed in Table 39.

Table 39 - pH values measured after reconstitution

Final Formulation Variant	pH after reconstitution
#1	7.33
#2	7.96
#1d	7.29
#2d	8.04
#C	6.90

pH values were in the expected range.

4.6 Aggregation by SE-HPLC

The aggregation status of GM-CSF samples after reconstitution with 0.33 mL WFI was evaluated by SE-HPLC. For reference, GM-CSF working standard (see Figure 16) as well as current formulation (lyophilized drug product “control”, #C containing HSA, see Figure 18) were analyzed in parallel. A representative chromatogram of the final formulation variants (exemplarily: #1) is shown in Figure 17.

For better comparability, separate SE-HPLC analytics on the drug substance material itself (GM-CSF Control Sample 3) were performed and results were related to a number of reference samples (results of these drug substance comparability analytics are summarized in section 6 of this Example).

The freshly thawed GM-CSF working standard showed a small peak of 0.36% relative peak area eluting after the main peak (99.64% relative peak area). The eluting order indicated a smaller Mw of those “fragments” than of the GM-CSF molecule. All reconstituted drug product

samples showed the same peak pattern of one peak of larger aggregates eluting before the main peak and one peak of smaller fragments eluting after the main peak.

The aggregate status of the existing commercial formulation #C could not be determined due to the HSA peak masking any potential GM-CSF aggregates. The exemplary chromatogram shown in Figure 18 should be considered informational only. The relative main peak areas detected for each individual sample are listed in Table 40

Table 40 - Relative main peak areas detected by SE-HPLC (aggregate status)

Variant	**Rel. main peak area**
GM-CSF Control Sample 1 (SEQ ID NO: 3)	99.64 %
GM-CSF Control Sample 3 (SEQ ID NO: 3)	(See section 6)
Final Formulation Variant #1	98.75 %
Final Formulation Variant #2	99.07 %
Final Formulation Variant #1d	99.19 %
Final Formulation Variant #2d	98.24 %

4.7 Degradation by RP-HPLC

The degradation (chemical purity) of the samples after reconstitution with 0.33mL WFI was evaluated by RP-HPLC. For reference, GM-CSF Control Sample 2 (see Figure 19) as well as lyophilized existing commercial formulation (lyophilized “control” formulation, #C, containing HSA, see Figure 21) were analyzed in parallel. A representative chromatogram of the developed final formulation variants (exemplarily: #1) is shown in Figure 20.

For better comparability, separate RP-HPLC analytics on the drug substance material itself (GM-CSF Control Sample 3) were performed and results were related to a number of reference samples (results of these drug substance comparability analytics were summarized in section 6 below).

The impurity peak profile in all reconstituted GM-CSF drug product final formulation variants #1, #2, #1d, #2d as well as in the working standard was the same and as shown in Figure 19 and

Figure 20. Peaks eluting before the main peak represented fragments that are more hydrophilic than GM-CSF whereas the peak eluting after the main peak represented fragments that are more hydrophobic than GM-CSF.

Likewise as for SE-HPLC, the chemical degradation of GM-CSF in the existing commercial formulation #C could not be determined as potential impurity peaks get masked by the HSA peak. Again, the respective chromatographic data should be considered informational only.

The relative main peak areas detected for each individual sample are listed in Table 41.

Table 41 - Relative main peak areas detected by RP-HPLC (chemical degradation)

Variant	**Rel. main peak area**
GM-CSF Control Sample 1	98.77 %
GM-CSF Control Sample 3	(see section 6)
Final Formulation Variant #1	97.95 %
Final Formulation Variant #2	97.83 %
Final Formulation Variant #1d	97.73 %
Final Formulation Variant #2d	97.65 %

4.8 Nano-particle analysis by DLS

To simplify the complex set of data generated with the DLS plate reader, three ranges of particle sizes were defined (i.e. 0.1-10 nm, 10-100 nm, and 100-1000 nm) and the mass fraction of molecules covered by the specific range was calculated. Table 42 shows the mean radius measured in the specific range as well as the related mass fraction.

Table 42 - Nano-particle size and distribution determined by DLS. Reference = GM-CSF Control Sample 1. “C” = lyophilized“control” formulation

Particle size range	Variant	Mean particle radius [nm]	Mean mass fraction [%]
0.1 – 10 nm	Reference	2.3	99.7
	Final Formulation Variant #1	1.4	99.6

	Final Formulation Variant #2	1.9	99.4
	Final Formulation Variant #1d	1.9	98.8
	Final Formulation Variant #2d	2.3	98.1
	#C	4.3	95.2
10 – 100 nm	Reference	36	0.10
	Final Formulation Variant #1	35	0.15
	Final Formulation Variant #2	67	0.45
	Final Formulation Variant #1d	61	0.80
	Final Formulation Variant #2d	48	1.5
	#C	0	0
100 – 1000 nm	Reference	385	0.03
	Final Formulation Variant #1	303	0.25
	Final Formulation Variant #2	164	0.15
	Final Formulation Variant #1d	323	0.45
	Final Formulation Variant #2d	347	0.50
	#C	159	4.8

In almost all samples as well as the working standard (reference) the vast majority of particles by mass (i.e. > 98 % in average) was 0.1-10 nm large, very comparable to the results obtained of selected samples measured after forced degradation study (see separate report in

Example3). Merely the existing commercial formulation #C showed a significantly higher content of larger particles (> 100 nm) of ~4.8 % in average. The small differences between the newly developed final formulation variants #1, #2, #1d, #2d, however, should not be overrated considering the variance of the method.

With a molecular weight of 14.6 kDa GM-CSF is a rather small molecule where the mean particle radius would be expected in the measured range between 0.1-10 nm, which seems to correlate well with the high amount of mass fraction detected in this range.

5. Conclusions

In this Example, the lyophilization feasibility of four final formulation variants for a lyophilized GM-CSF drug product was tested using a conservative lyo cycle (primary drying at -10 °C and 100 µbar). For reference and comparability a placebo formulation (#2v) was freeze dried in the same run. Table 43 lists the tested variants.

Table 43 - Formulation variants tested for lyophilization feasibility in Example 5.

Final Formulation Variant #	**Composition of liquid bulk drug substance**	**Composition of lyophilized drug product per vial**	**Comment**
1	0.30 mg/mL, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 7.3	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 7.3)	Regular formulation #1 fill volume: 330 µL, batch size: 160 vials
1d	0.15 mg/mL, 5 mM K-phosphate, 130 mM sucrose, 0.05 % (w/v) poloxamer 188, pH 7.3	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 7.3)	Diluted formulation #1 fill volume 660 µL batch size: 60 vials
2	0.30 mg/mL, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188,	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to	Regular formulation #2 fill volume: 330 µL batch size: 160 vials

	pH 8.1	pH 8.1)	
2d	0.15 mg/mL, 5 mM K-phosphate, 130 mM sucrose, 0.05 % (w/v) poloxamer 188, pH 8.1	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 8.1)	Diluted formulation #2 fill volume: 660 µL batch size: 60 vials
2v	10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 8.1	0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 8.1)	Vehicle/placebo formulation #2 fill volume: 330 µL batch size: 40 vials

After lyophilization, the products were analyzed with respect to:

- visual appearance, documented by macro photography
- residual water content using a generic Karl-Fischer oven method (in duplicate)
- reconstitution behavior (using 0.33 mL WFI)
- pH measurement after reconstitution
- aggregation status by SE-HPLC
- degradation profile by RP-HPLC
- nano-particle content by DLS

For reference and comparability, a working standard (reference) as well as the existing commercial formulation (hereafter variant #C) were analyzed in parallel using the same methods.

For better comparability, separate SE-HPLC and RP-HPLC analytics on the drug substance material itself (GM-CSF Control Sample 3) were performed and results were related to a number of reference samples (results of these drug substance comparability analytics were summarized in section 6 below).

Visual appearance:

All lyophilized formulation samples showed acceptable cakes with merely mild shrinkage commonly observed with sucrose cakes. No larger defects could be detected. The existing commercial formulation (lyophilized “control” formulation; #C) did not show shrinkage as expected for a mannitol cake.

Residual water content:

All measured residual water values in the lyophilized development samples were well below 1%. The existing commercial formulation (lyophilized "control" formulation; #C), however, showed a significantly higher content of water of 0.6 %.

Reconstitution behavior:

The addition of 0.33 mL WFI and subsequent immediate and gentle shaking lead to a complete dissolution of all final formulation variants in less than 10 seconds. The exiting commercial formulation (lyophilized "control" formulation; #C) dissolved slightly slower than the final formulation variants.

pH values after reconstitution:

After reconstitution with 0.33 mL WFI, all measured pH values were within a range of ±0.2 pH units of the target value (pH 7.3 or 8.1, respectively). In fact, only final formulation variant #2 showed more than 0.1 pH units of deviation (-0.14 pH units). In the existing commercial formulation (lyophilized "control" formulation; #C) a pH value of 6.9 was measured (stored for 2 years and 7 months; at release, pH measured was 7.0).

Aggregation status by SE-HPLC:

All final formulation vairants showed an aggregate/fragment content of less than 2 %. The reference working standard had a content of 0.36 %, whereas the aggregation status of the existing commercial formulation (lyophilized "control" formulation; #C) could not be determined due to HSA masking potential peaks in the chromatogram.

Degradation by RP-HPLC:

All final formulation variants showed a GM-CSF purity of more than 97.5 %. The reference working standard had a purity of 98.8 %, whereas the aggregation status of the existing commercial formulation (lyophilized "control" formulation; #C) could not be determined due to HSA masking potential peaks in the chromatogram.

Nano-particle analysis by DSL:

In all final formulation variants, the vast majority (>98 %) of detected particles (with respect to mass) showed a radius of ~2 nm. The working standard showed a fraction of 99.7% in this particle size range. The existing commercial formulation (lyophilized "control" formulation; #C), however, showed only 95.2% of particles by mass in the range of 0.1-10 nm, whereby the mean radius was detected as more than double as large as in the final formulation variants with 4.3nm. The larger radius is probably due to HSA, whereas the higher content of larger particles may be a sign of aggregation.

Example 6

1. Scope and objectives

The objective of this development work was to evaluate GM-CSF Control Sample 3 (Lot: 170118/1167/095; SEQ ID NO: 3), used for Example 5, with regard to comparability to GM-CSF Control Sample 2 (Lot: 160126/1089/082). In particular, this study aimed to discover whether the impurity profile of different batches of GM-CSF are consistent, to ensure that new batches of GM-CSF do not introduce impurities.

GM-CSF Control Sample 3 was not analyzed on SE-HPLC and RP-HPLC before lyophilization in Example 5. However, this analysis was found to be missing in order to relate obtained results of Example 5 against a non-freeze-dried reference of GM-CSF Control Sample 3.

The following samples were to be tested in a comparability test per SE-HPLC method including MALS determination (Example 6; analysis on Superdex column) and RP-HPLC analysis (Example 7):

- Reference injection of GM-CSF Control Sample 2 used for Examples 1-5.
- Reference injection of GM-CSF Control Sample 1(Lot: 150904/1064/71)
- Injection of GM-CSF Control Sample 3(Lot. No. 170118/1167/095) used for Example 5, incl. MW determination of each peak for peak identification
- Attempt for generation of a positive control of GM-CSF Control Sample 3by e.g. thermal stress or light stress, in order to relate results against
- Lyo sample #1 and #2 from Example 5 (final formulations #1 and #2, respectively; one spare vial each)
- Corresponding liquid placebo solution of sample #1 and #2 (freshly prepared)
- One sample of Molgramostim from the manufacturer of an existing commercial formulation of Molgramostim.

The additional analytics were to be used to evaluate the results obtained in Example 5 further.

2. Materials and Apparatus

2.1 Substances and materials

Table 44 – Substances and materials.

Substances	**Lot no.:**	**Manuf. / Suppl.**	**Order no.:**
GM-CSF Control Sample 1 (SEQ ID NO: 3)	150904/1064/71	-	-

GM-CSF Control Sample 2 (SEQ ID NO: 3)	160126/1089/082	-	-
GM-CSF Control Sample 3 (SEQ ID NO: 3)	170118/1167/095	-	-
Molgramostim (rHuGM-CSF)	CS2015A05	-	-
GM-CSF lyophilizate #1 (final formulation variant #1)	TGWP7b/1-170814	PJP	-
GM-CSF lyophilizate #2 (final formulation variant #2)	TGWP7b/2-170814	PJP	-
Purified water (≤ 0.2 µmS/cm, < 10 ppb TOC)	-	PJP	-
Kolliphor® P 188, Poloxamer, Ph. EUR.	WPDH566B	BASF	50259527
ortho-phosphoric acid 85 %, EMPROVE® exp. Ph. Eur.	K47168363542	Merck Chemicals	100563
Sodium chloride, EMPROVE® exp Ph. Eur. BP, USP	K45515600	Merck Chemicals	106400
Sucrose, EMPROVE® exp. Ph.Eur	K42784453205	Merck Chemicals	107653
Sodium hydroxide solution, 30 % w/w pure, pharma grade	0000862568	AppliChem	144320.1211
Potassium hydroxide, EMPROVE® exp Ph Eur	B03389302	Merck Chemicals	105032
Trifluoroacetic acid, ≥ 99.9 %, for peptide synthesis	245230492	Carl Roth GmbH	P088.1
Acetonitrile, Chromasolv gradient grade	SZBG041AV	Sigma-Aldrich	34851
Materials			
Minisart® PES 0.1 µm syringe filter	70681103	Sartorius stedim	16553-K
Jupiter 5 µm C18 (300 Å; 250 × 4.6 mm)	H16-035225	Phenomenex	00G-4053-E0

Superdex™ 200 Increase 10/300 GL	10246668	GE Healthcare	28-990o9-44

2.2. Apparatus

Apparatus used in Example 6 and Example 7 are the same as listed in section 0 of Example 5.

3. Methods

3.1 Preparation of buffers and samples

Formulation buffers (placebo solution)

The preparation of the formulation buffers for blanking was described in section 3.1 of Example 5.

Sample solutions

GM-CSF containing DS solutions were diluted to a concentration of 0.3 mg/mL using elution buffer (for SEC-MALS) or dilution buffer (for RP-HPLC), respectively. Lyophilizates were reconstituted prior to analysis using 0.33 mL WFI.

3.2 Analytical methods

Analytical methods used in Example 6 and Example 7 were described in sections 0 and 0 of Example 5.

4. Results and discussion

4.1 SEC-MALS

In the following section, chromatogram overlays of different samples are shown and commented on. Values for aggregate/fragment peak areas as well as main peak areas are listed in Table 45 at the end of this section.

Peak identification using a MALS detector

Peak identification was performed using a stressed “new” BDS sample of lot -/095 (Control Sample 3). The BDS was stressed at 55 °C for 60 hours before the aggregate status was determined by SEC-MALS. The chromatogram is shown in Figure 22. An aggregate peak at RT=29.3 minutes could be identified with a Mw of about 49 kDa. The Mw in the main peak was determined as 14 kDa. The MALS and UV signals of suspected fragments was too low for the proper calculation of molecular weight. The molecular weight displayed by the red dots at around RT=37.5 minutes does not represent real occurrences and may not be interpreted as such.

Comparison of different lots of GM-CSF

The three different lots of GM-CSF BDS (150904/1064/71, Control Sample 1; 160126/1089/082, Control Sample 2; 170118/1167/095, Control Sample 3) were compared using the described SE-HPLC method for aggregate status determination. An overlay of the three chromatograms is shown in Figure 23. Due to slight variations in GM-CSF concentration, the peak height of the three main peaks is not completely aligned. With respect to aggregates and fragments, however, no significant difference could be detected between the lots.

Comparison of lot -/095 with Molgramostim

The BDS material used for Examples 5 and 6 (lot. no. 170118/1167/095, Control Sample 3) was compared to Molgramostim (lot. no. CS2015A05) using the described SE-HPLC method for aggregate status determination. An overlay of the two chromatograms is shown in Figure 24. No significant difference between the two samples could be detected by SE-HPLC.

Comparison of lot -/095 with reconstituted lyophilizates from Example 5

The BDS material used for Examples 5 and 8 (lot. no. 170118/1167/095, Control Sample 3) was compared to reconstituted lyophilizates produced in Example 5 (TGWP7b/1-170814, final formulation variant #1; and TGWP7b/2-170814, final formulation variant #2) using the described SE-HPLC method for aggregate status determination. An overlay of the three chromatograms is shown in Figure 25. A slight increase in aggregate and fragment content in the lyophilized samples is visible. Final formulation variant #2 (TGWP7b/2-170814) has slightly less impurity content than final formulation variant #1 (TGWP7b/1-170814).

Comparison of reconstituted lyophilizates from Example 5 with their respective placebo formulation

The reconstituted lyophilizates produced in Example 5 (Table 44; TGWP7b/1-170814, final formulation variant #1; and TGWP7b/2-170814, final formulation variant #2) were compared to their respective placebo formulations using the described SE-HPLC method for aggregate status determination. Overlays are shown in Figure 26 and Figure 27. A small part of the fragmentation peak at RT = 38.5 minutes seems to derive from the formulation buffer (salt peak). The method is not well suited for fragment quantification as salts and fragments elute together. Using a different SE-HPLC column better suited for small proteins could lead to an earlier elution of GM-CSF allowing for a better distinction between salts and fragments. The aggregate peak visible in the lyophilizates, however, is well distinguishable from the baseline.

Comparison of lot -/095 of drug substance to positive control

A positive control was produced by incubating "new" BDS of lot -/095 (Control Sample 3) at 55 °C for 60 hours. The positive control was compared to freshly thawed "new" BDS of the same batch using the described SE-HPLC method for aggregate status determination. An overlay of the two chromatograms is shown in Figure 28.

The increasing amount of aggregates induced by stressing the BDS thermally is well visible and distinguishable from the main GM-CSF peak. Quantification of aggregates should not be an issue. Increasing fragmentation is also visible but the fragments elute together with the salts of the buffer which negatively affects quantification.

Summary of relative peak areas of all samples

Table 45 - Relative peak areas determined by SE-HPLC

Sample	**Rel. main peak area**	**Rel. aggregate peak area**	**Rel. fragment peak area**
BDS lot -/71 (Control Sample 1)	99.82 %	-	0.18 %
BDS lot -/082 (Control Sample 2)	99.88 %	-	0.12 %
BDS lot -/095 (Control Sample 3)	99.87 %	-	0.13 %
Molgramostim	99.90 %	0.01 %	0.09 %
Lyophilizate #1 (final formulation variant #1)	99.22 %	0.32 %	0.45 %
Lyophilizate #2 (final formulation variant #2)	99.44 %	0.27 %	0.30 %

6.4.2 RP-HPLC

In the following section chromatogram overlays of different samples are shown and commented on. Values for impurity peak areas as well as main peak areas are listed in Table 46 at the end of this section.

Comparison of different lots of drug substance from Wacker

The three different lots of GM-CSF BDS (150904/1064/71, Control Sample 1; 160126/1089/082, Control Sample 2; and 170118/1167/095, Control Sample 3) were compared using the

described RP-HPLC method for purity analysis. An overlay of the three chromatograms is shown in Figure 29. In BDS lot -/095 (Control Sample 3) a small peak (0.16 %) at 8.6 minutes is visible that was not detected in the two other lots. Overall the purity of the three lots seemed comparable. Numeric values are given in Table 46at the end of this section.

Comparison of lot -/095 with Molgramostim

The BDS material used for Examples 5 and 8 (lot. no. 170118/1167/095, Control Sample 3) was compared to Molgramostim (lot. no. CS2015A05) using the described RP-HPLC method for purity analysis. An overlay of the two chromatograms is shown in Figure 30. The impurity peak at 8.6 minutes does not appear in the Molgramostim sample. Other than that no significant difference between the two samples could be detected by RP-HPLC.

Comparison of GM-CSF drug substance lot -/095 with reconstituted GM-CSF drug product lyophilizates from Example 5

The BDS material used for Example 5 (lot. no. 170118/1167/095, Control Sample 3) was compared to reconstituted drug product lyophilizates produced in Example 5 (TGWP7b/1-170814, final formulation variant #1; and TGWP7b/2-170814, final formulation variant #2) using the described RP-HPLC method for purity analysis. An overlay of the three chromatograms is shown in Figure 31.

A slight decrease in purity in the lyophilized samples is visible compared to the BDS. The impurity peak at 8.6 minutes, however, is not apparent after lyophilization and reconstitution anymore. It may get further degraded to impurities that are either not detectable by this method or that add to one of the other impurity peaks. The two lyophilizates do not show significant differences compared to each other.

Comparison of reconstituted GM-CSF drug product lyophilizates from Example 5 with their respective placebo formulation

The reconstituted drug product lyophilizates produced in Example 5 (TGWP7b/1-170814, final formulation variant #1; and TGWP7b/2-170814, final formulation variant #2) were compared to their respective placebo formulations without drug substance GM-CSF using the described RP-HPLC method for purity analysis. Overlays are shown in Figure 32 and Figure 33.

None of the detected impurity peaks are visible in the corresponding placebo solutions. Thus, all impurities detected by RP-HPLC can be considered product related.

Comparison of drug substance lot -/095 to positive control

A positive control was produced by incubating BDS of lot -/095 (Control Sample 3) at 60 °C for 18 hours. The positive control was compared to freshly thawed BDS of the same lot using the described RP-HPLC method for purity analysis. An overlay of the two chromatograms is shown in Figure 34.

Heat stressing the BDS lead to an increase in relative impurity peak areas. Impurity peaks eluted at 8.6, 19.2, 20.5, 24.8, 26.9, and 28.6 minutes with more peaks evolving between 20.5 and 24.8 minutes as well as after the peak at 28.6 minutes.

Summary of relative peak areas of all samples

Table 46 - Purity determined by RP-HPLC

Sample	Rel. main peak area
BDS lot -/71 (Control Sample 1)	98.66 %
BDS lot -/082 (Control Sample 2)	98.89 %
BDS lot -/095 (Control Sample 3)	98.37 %
Molgramostim	99.02 %
Lyophilizate #1 (final formulation variant #1)	97.66 %
Lyophilizate #2 (final formulation variant #2)	97.57 %

6.5 Conclusions

The three GM-CSF Control Samples are comparable to each other as well as to the existing commercial Molgramostim drug substance, with respect to aggregates/fragments (SE-HPLC) as well as impurity profile (RP-HPLC). Merely GM-CSF Control Sample 3 shows a small impurity peak in RP-HPLC chromatograms at about RT = 8.6 minutes that is not visible in any of the other lots or the lyophilized DP. While still detectable in the stressed positive control, it is not clear if it degrades to an undetectable impurity during the drug product production process or if it adds to one of the other impurity peaks.

Between GM-CSF Control Samples 1-3 and the existing commercial Molgramostim, the latter showed the highest purity by RP-HPLC (99.02 %), whereas GM-CSF Control Sample 3 showed the lowest with 98.37 %. No significant differences could be detected by SE-HPLC.

Analyzing a positive control by SEC-MALS revealed one aggregate peak with a Mw of ~49 kDa, the main GM-CSF peak with ~14 kDa, and a fragmentation peak. The UV absorbance of the fragments, however, was not high enough for the determination of the Mw. Furthermore, it has to be noted that the method is not well suited for the quantification of molecules smaller than GM-CSF (i.e. fragments) as these elute partly together with the salts of the buffer.

Example 7

Comparison of lyophilizates from Example 5 with an existing commercial formulation by SE-HPLC and RP-HPLC

The lyophilizates obtained in Example 5 (GM-CSF lyophilizate #1 (final formulation variant #1)and GM-CSF lyophilizate #2 (final formulation variant #2)) were compared to an existing commercial formulation by overlaying the respective chromatograms. The chromatograms obtained by SE-HPLC analysis are shown in Figure 35.

The main peak area detected in the existing commercial formulation was between 10% and 15% higher than in the two lyophilizates obtained in Example 5. This may be due to an overage applied by the manufacturer to overcome extraction issues (i.e. not the complete liquid volume can be extracted after reconstitution). The peak pattern, however, is not comparable as the existing commercial formulation contains HSA and its aggregates masking the relevant retention time range for GM-CSF aggregates.

The chromatograms obtained by RP-HPLC are shown in Figure 36.

As for RP-HPLC, the main peak area detected in the existing commercial formulation was higher (up to almost 20% here) than in the lyophilizates obtained in Example 5. The relative differences between SE-HPLC and RP-HPLC may be due to the HSA peak interfering with the GM-CSF peak differently in the two methods. Furthermore, the main peak eluted about one minute earlier than in the two lyophilizates. This is a phenomenon commonly observed in RP-HPLC deriving from slight differences in composition of the elution buffer. The large HSA peak in the current drug product again masks a broad range of retention time making a proper evaluation of impurities impossible by this method.

Example 8

1. Scope and objective

Example 8 was aimed to evaluate the stability of the four different final formulation variants of lyophilized GM-CSF drug product. Lyophilized samples that entered this study were

manufactured using the same procedure as in Example 5, using a conservative, non-optimised lyo-cycle.

Lyo samples obtained in Example 5 were stored at 25 °C and 40 °C, respectively, for up to 12 weeks (pull points were set to T8 weeks and T12 weeks). Formulation variants stored are listed in Table 47.

Table 47 - Formulation variants tested for accelerated stability in Example 8.

Final Formulation Variant #	**Composition of liquid bulk drug substance**	**Composition of lyophilized drug product per vial**	**Comment**
1	0.30 mg/mL, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 7.3	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 7.3)	Regular formulation #1 fill volume: 330 µL batch size: 160 vials
1d	0.15 mg/mL, 5 mM K-phosphate, 130 mM sucrose, 0.05 % (w/v) poloxamer 188, pH 7.3	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 7.3)	Diluted formulation #1 fill volume: 660 µL batch size:: 60 vials
2	0.30 mg/mL, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 8.1	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 8.1)	Regular formulation #2 fill volume: 330 µL batch size: 160 vials
2d	0.15 mg/mL, 5 mM K-phosphate, 130 mM sucrose, 0.05 % (w/v) poloxamer 188, pH 8.1	0.10 mg GM-CSF 0.32 mg phosphoric acid 29.4 mg sucrose 0.33 mg poloxamer 188 (potassium hydroxide to pH 8.1)	Diluted formulation #2 fill volume: 660 µL batch size: 60 vials

Samples per time point and temperature were analyzed immediately after lyophilization (T0, per Example 5) and after storage periods, by the following methods:

- visual appearance, documented by macro photography
- reconstitution behavior (using 0.33 mL WFI)
- pH measurement after reconstitution
- residual water content using a generic Karl-Fischer oven method (in duplicate)
- aggregation status by SE-HPLC
- chemical degradation by RP-HPLC
- nano-particle analysis by DLS

2. Materials and Apparatus

2.1 Substances and Materials

Table 48 – Substances and materials

Substances	**Lot no.:**	**Manuf. / Suppl.**	**Order no.:**
GM-CSF Control Sample 1	150904/1064/71	-	-
GM-CSF lyophilizate #1 (final formulation variant #1)	TGWP7b/1-170814	PJP	-
GM-CSF lyophilizate #2 (final formulation variant #2)	TGWP7b/2-170814	PJP	-
GM-CSF lyophilizate #1d (final formulation variant #1d)	TGWP7b/1d-170814	PJP	-
GM-CSF lyophilizate #2d (final formulation variant #2d)	TGWP7b/2d-170814	PJP	-
Purified water (≤ 0.2 µmS/cm, < 10 ppb TOC)	-	PJP	-
ortho-phosphoric acid 85 %, EMPROVE® exp. Ph. Eur.	K47168363542	Merck Chemicals	100563
Sodium chloride, EMPROVE® exp Ph. Eur. BP, USP	K45515600	Merck Chemicals	106400
Sodium hydroxide solution, 30 % w/w pure, pharma grade	0000862568	AppliChem	144320.1211

Potassium dihydrogen phosphate, ≥ 98 %, Ph.Eur., USP, BP	261168601	Carl Roth GmbH	P018
di-Sodium hydrogen phosphate, ≥ 98 %, Ph.Eur	373201802	Carl Roth GmbH	T876
Trifluoroacetic acid, ≥ 99.9 %, for peptide synthesis	245230492	Carl Roth GmbH	P088.1
Acetonitrile, Chromasolv gradient grade	SZBG041AV	Sigma-Aldrich	34851
Materials			
Jupiter 5 µm C18 (300 Å; 250 × 4.6 mm)	H16-035225	Phenomenex	00G-4053-E0
Superdex™ 200 Increase 10/300 GL	10246668	GE Healthcare	28-990o9-44

2.2 Apparatus

The following apparatus and equipment was used:

Purified water supply

- Manufacturer: Siemens
- Type: Ultra Clean UV UF TM

HPLC Equipment

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: 1100 Series
- Degasser G1322A
- BinPump G1312A
- ALS G1329A
- ALSTherm G1330A
- ColCom G1316A
- DAD G1315A

Camera Equipment

- Manufacturer: Canon (Tokio, Japan)
- Body: EOS 550D/EOS 600D
- Lens: EFS 18-55mm, DGMacro 105mm 1:2.8

DLS plate reader

- Manufacturer: Wyatt Technology Corporation (Santa Barbara, CA, USA)
- Type: Dynapro plate reader II

Additional Laboratory Equipment

- Analytical balance (Kern)
- Magnetic stirrer (IKA Werke)
- pH meter (Mettler Toledo)
- Volumetric pipets (Gilson)
- Multistep Pipettes: Brand, HandyStep
- Autoclave: Systec, DX65
- Drying oven: Memmert, 500
- Orbital shaker: Wisd Laboratory Instruments, WiseShake SHO-1D

Laminar flow microbiological class 2 work bench: BDK, UVF 6.18S

All equipment that was used undergoes regular calibration and qualification according to the valid qualification and maintenance plan.

3. Methods

3.1 Analytical methods

3.1.1 SE-HPLC (size exclusion chromatography)

Chromatographic parameters:

Mobile phase: 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0
Flow rate: 0.5 mL/min
Temperature: ambient
UV-detection: 214 nm and 280 nm
Injection volume: 100 µL at 0.3 mg/mL GM-CSF
Sample temperature: 4 ± 2 °C

Buffer and sample preparation:

Table 49 – Buffer and sample preparation.

Buffer/solution	Composition/Description	Preparation procedure
Mobile phase	0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0	11.53 g of 85 % (w/w) phosphoric acid and 5.84 g of sodium chloride were topped with 900 mL with purified water in a 1000 mL glass beaker. The pH was adjusted to 7.0

		with sodium hydroxide before the volume was filled up to 1000 mL with purified water.
Reference solution	0.3 mg/mL GM-CSF in mobile phase	GM-CSF Control Sample 1 was diluted to 0.3 mg/mL with mobile phase.
Sample solution	Reconstituted lyophilizate	Lyophilizates were reconstituted with 0.33 mL WFI prior to analysis.

3.1.2 RP-HPLC (reversed-phase chromatography)

Chromatographic parameters:

Mobile phase A: 0.1 % TFA in purified water

Mobile phase B: 10 % purified water + 0.1 % TFA in ACN

Flow rate: 1.2 mL/min

Temperature: 50 ± 2 °C

UV-detection: 214 nm

Injection volume: 100 µL at 0.5 mg/mL

Sample temperature: 4 ± 2 °C

Gradient:

Table 50 - Gradient

Time [min]	Pump B conc. [%]
0.10	36
30	56
35	100
45	100
50	36
60	36

Buffer and sample preparation:

Table 51 – Buffer and sample preparation.

Buffer/solution	Composition/Description	Preparation procedure
Mobile Phase A	0.1 % TFA in purified water	1 mL of trifluoroacetic acid was mixed with

		1000 mL of purified water.
Mobile Phase B	10 % purified water + 0.1 % TFA in ACN	1 mL of trifluoroacetic acid was mixed with 100 mL of purified water and 900 mL of acetonitrile.
Dilution buffer	0.05 M phosphate buffer, pH 7.0	2.63 g of potassium dihydrogen phosphate and 4.33 g of disodium hydrogen phosphate were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.0 using sodium hydroxide and the volume was filled up to 1000 mL with purified water.
Reference solution	0.3 mg/mL GM-CSF in dilution buffer	GM-CSF Control Sample 1 was diluted to 0.3 mg/mL with dilution buffer.
Sample solution	Reconstituted lyophilizate	Lyophilizates were reconstituted with 0.33 mL WFI prior to analysis.

3.1.3 Nano-particle analysis by DLS (dynamic light scattering)

The principle of the Wyatt DynaPro Plate reader II is based on dynamic light scattering, and is described in section 3.1 of Example 3.

Sample preparation:

Each sample constituted a 2R vial with lyophilized GM-CSF formulation with a target GM-CSF amount of 0.10 mg per vial. After reconstitution with 0.33 mL WFI samples were filled into the wells of the measurement plate by portions of 30 µL and subsequently overlaid with silicone oil to avoid evaporation of liquid. No further treatment was applied before measurement.

3.1.4 Visual appearance of the lyophilizate

Lyophilizate-containing vials were examined visually. Photographs were taken for documentation.

3.1.5 Karl-Fischer oven method

The residual moisture of the samples was determined via Karl Fischer titration using a 756 Karl Fischer coulometer equipped with a 774 oven sample processor (Metrohm). For each measurement one lyophilizate of each sample (~ 20 - 30 mg of a lyophilizate) was transferred in a Karl-Fischer glass vial. The exact weight of the lyophilizate was documented. The vial was sealed with a crimp cap and transferred into the oven of the Karl Fischer coulometer which was

heated to 100 °C. The septum of the crimp cap was penetrated by an injection needle, and the generated water vapour was directly transferred into the titration chamber of the Karl Fischer coulometer via dry nitrogen flow. Empty glass vials were used as blank correction. Samples were analyzed in duplicate.

3.1.6 Reconstitution

The vials were reconstituted by the addition of 330 µL WFI using a volumetric pipette after removing the cap and stopper from the vial, according to the same method as described in section 3.3.8 of Example 5. Subsequently the vial was gently shaken until complete dissolution of the lyo cake.

3.1.7 pH measurement after reconstitution

pH values were measured directly in the vial after reconstitution using a calibrated pH meter (Mettler Toledo).

4. Results and discussion

The lyophilized samples (40 lyophilized vials per final formulation variant) of each of the four final formulation variants as listed below were stored at 25 °C and 40 °C for up to 12 weeks according to Table 52 and Table 53 below. 5 lyophilized samples were taken per sampling point (T0 from Example 5) of this study at T8 weeks, and at T12 weeks.

Table 52 – Variants used

Final Formulation Variant #	**Formulation (final formulation)**	**Fill volume per vial**
#1	0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, pH 7.3, 0.1 % P188	0.33 mL
#2	0.3 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, pH 8.1, 0.1 % P188	0.33 mL
#1d	0.15 mg/mL GM-CSF, 5 mM K-phosphate, 130 mM sucrose, pH 7.3, 0.05 % P188	0.66 mL
#2d	0.15 mg/mL GM-CSF, 5 mM K-phosphate, 130 mM sucrose, pH 8.1, 0.05 % P188	0.66 mL

Table 53 –Storage conditions

Presentation: Set-up "C" (4 variants), set-up per variant			
	T0	**T8 weeks**	**T12 weeks**
25 °C	(see Example 5)	5 vials (+ 5 spare vials)	5 vials (+ 5 spare vials)
40 °C		5 vials (+ 5 spare vials)	5 vials (+ 5 spare vials)
sum	-	20 vials	20 vials
total			**40 vials**

Samples per time point and temperature were analyzed immediately after lyophilization (T0, per Example 5), and after storage periods with the methods as reported in the following.

4.1 Visual appearance of the lyo cakes

Directly after lyophilization (T=0), after T=8 weeks, as well as after T=12 weeks lyophilizates were inspected visually and photographs were taken of representative vials. Photographs are shown in Figure 37, Figure 38, and Figure 39, respectively. Macroscopically no significant differences could be identified throughout all time points and storage temperatures.

4.2 Residual water content

The residual water content in the lyophilizates was determined using a generic Karl-Fischer oven method (100 °C). The results are listed in Table 54.

Table 54 - Residual water content by Karl-Fischer oven method.

Final Formulation Variant	**T=0**	**T=8 weeks**		**T=12 weeks**	
		25 °C	**40 °C**	**25 °C**	**40 °C**
#1	0.3 %	0.6 %	0.7 %	0.6 %	0.7 %
#2	0.5 %	0.6 %	0.9 %	0.6 %	0.7 %
#1d	0.2 %	0.5 %	0.7 %	0.5 %	0.7 %
#2d	0.2 %	0.5 %	0.7 %	0.5 %	0.7 %

All measured residual water values in the lyophilized samples were well below 1 %. While stored the lyophilizates took up 0.3-0.5 percentage points of water which assumedly derived from the lyo stoppers. The increase in water content was slightly higher in the vials stored at 40 °C compared to the vials stored at 25 °C by approximately 0.1 percentage points. Between 8

and 12 weeks of storage no significant difference could be identified indicating that the majority of water was released from the stoppers in the first 8 weeks already.

4.3 Reconstitution behavior

Reconstitution was performed by addition of 0.33 mL WFI and subsequent immediate and gentle shaking. The complete dissolution of the lyo cake of all final formulation variants #1, #2, #1d, #2d was achieved after about 10 seconds, regardless of storage time or temperature.

4.4 pH after reconstitution

After reconstitution with 0.33 mL WFI the pH value of each variant was determined. The measured values are listed in Table 55.

Table 55 - pH values measured after reconstitution.

Final Formulation Variant	**T=0**	**T=8 weeks**		**T=12 weeks**	
		25 °C	**40 °C**	**25 °C**	**40 °C**
#1	7.33	7.23	7.28	7.22	7.35
#2	7.96	8.09	7.99	8.02	8.10
#1d	7.29	7.39	7.31	7.32	7.35
#2d	8.04	8.11	8.08	8.03	8.09

Measured pH values were within the expected range.

4.5 Aggregation by SE-HPLC

The aggregation status of GM-CSF samples after reconstitution with 0.33 mL WFI was evaluated by SE-HPLC. For reference, GM-CSF working standard was analyzed in parallel. A representative chromatogram overlay of final formulation variant #1 after storage at 40 °C for 8 weeks and 12 weeks, respectively, is shown in Figure 40.

The same peak pattern consisting of an aggregate peak at RT=28 minutes, the main peak at RT=33 minutes, and a fragments peak at 38 minutes could be observed for all analyzed samples. The detected relative main peak areas are listed in Table 56.

Table 56 - Relative main peak areas detected by SE-HPLC (aggregate status)

Variant	**T=0**	**T=8 weeks**		**T=12 weeks**	
		25 °C	**40 °C**	**25 °C**	**40 °C**
Reference	99.6	-	-	-	-
#1	98.8	98.8	99.3	99.3	99.5
#2	99.1	99.2	99.0	99.6	99.7
#1d	99.2	98.7	99.0	99.6	99.5
#2d	98.1	99.0	99.0	99.3	99.6

For a graphic overview the listed main peak areas were processed in bar graphs shown in Figure 41 and Figure 42.

After 8 weeks of storage no significant difference in main peak area compared to T=0 could be identified. After 12 weeks of storage a slight increase in main peak area by about 0.5 percentage points could be detected for all variants and temperatures. This might be due to aggregates/fragments getting further degraded to species that are not detectable by this method anymore. No significant difference between the four variants could be identified.

4.6 Degradation by RP-HPLC

The degradation (chemical purity) of the samples after reconstitution with 0.33 mL WFI was evaluated by RP-HPLC. For reference, GM-CSF working standard was analyzed in parallel. A representative chromatogram overlay of final formulation variant #1 after storage at 40 °C for 8 weeks and 12 weeks, respectively, is shown in Figure 43.

The same peak pattern consisting of a peak at RT=19 minutes, followed by three small impurity peaks, the main peak at RT=25 minutes, and a peak at 27 minutes could be observed for all analyzed samples. The detected relative main peak areas are listed in Table 57.

Table 57 - Relative main peak areas detected by RP-HPLC (aggregate status).

	T=0	**T=8 weeks**		**T=12 weeks**	
Final Formulation Variant		25 °C	40 °C	25 °C	40 °C
Reference	98.8	-	-	-	-

#1	98.0	97.4	96.9	96.9	96.3
#2	97.8	97.5	96.5	97.0	96.4
#1d	97.8	97.3	97.0	97.1	96.1
#2d	97.7	97.2	96.7	96.9	96.3

For a graphic overview the listed main peak areas were processed in bar graphs shown in Figure 44 and Figure 45.

At 25°C storage temperature a decrease in main peak area by about 0.6 percentage points after 8 weeks and about 1.0 percentage point after 12 weeks compared to T=0 could be detected. At 40°C storage temperature the purity was overall slightly lower by about 0.5-1.0 percentage points. After 12 weeks at 25°C the purity was identified as roughly 97.0% in all variants, whereas at 40°C the purity was determined as 96.1-96.4%. No significant differences could be identified between the variants.

4.7 Nano-particle analysis by DLS

To simplify the complex set of data generated with the DLS plate reader, three ranges of particle sizes were defined (i.e. 0.1-10 nm, 10-100 nm, and 100-1000 nm) and the mass fraction of molecules covered by the specific range was calculated. Table 58 shows the mean radius measured in the specific range as well as the related mass fraction.

Table 58 - Nano-particle size and distribution determined by DLS. Ref = GM-CSF working standard.

Particle size range	**Final Formulation Variant**	**T=0**		**T=8 weeks**				**T=12 weeks**			
				25 °C		40 °C		25 °C		40 °C	
		Mean particle radius [nm]	Mean mass fraction [%]	Mean particle radius [nm]	Mean mass fraction [%]	Mean particle radius [nm]	Mean mass fraction [%]	Mean particle radius [nm]	Mean mass fraction [%]	Mean particle radius [nm]	Mean mass fraction [%]
0.1 – 10 nm	Ref	2.3	99.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	#1	1.4	99.6	1.6	97.9	1.7	99.0	0.5	99.4	2.1	93.8
	#2	1.9	99.4	1.8	96.7	1.3	99.2	0.5	99.7	0.9	99.9
	#1d	1.9	98.8	1.7	99.6	3.1	94.7	0.9	99.9	3.0	97.2
	#2d	2.3	98.1	0.9	99.5	0.5	99.7	2.3	97.3	2.2	97.9
10 – 100 nm	Ref	36	0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	#1	35	0.15	49.4	0.50	29.2	0.50	-	-	-	-
	#2	67	0.45	15.5	1.20	31.5	0.30	-	-	71.6	0.10
	#1d	61	0.80	-	-	50.1	3.00	53.3	0.10	43.1	1.80
	#2d	48	1.5	11.6	0.40	-	-	43.5	1.50	48.2	0.90
100 –	Ref	385	0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

1000 nm												
	#1	303	0.25	454	1.60	324	0.60	160	0.00	102	1.40
	#2	164	0.15	129	1.30	464	0.50	148	0.00	-	-
	#1d	323	0.45	105	0.40	357	2.30	-	-	425	1.00
	#2d	347	0.50	322	0.20	200	0.00	261	1.20	508	1.20

With a molecular weight of 14.6 kDa, GM-CSF is a rather small molecule where the mean particle radius would be expected in the measured range between 0.1-10 nm, which seems to correlate well with the high amount of mass fraction detected in this range in all samples.

Higher fluctuations as observed in some samples that showed less than 95 % of particles ranging between 0.1 and 10 nm seem to occur rather randomly and should not be overrated.

5. Discussions and conclusions

In Example 8, the stability of GM-CSF in four different lyophilized final formulation variants was evaluated after storage of the samples at 25°C and 40°C, respectively. Samples were analyzed right after lyophilization, after 8 weeks, and after 12 weeks of storage by the following methods.

Visual appearance, documented by macro photography:

The lyo cakes of all variants showed some light shrinkage already right after lyophilization. No significant change of the macroscopic appearance could be identified after storage at either temperature.

Reconstitution behavior (using 0.33 mL WFI):

Reconstitution of all samples was finished after about 10 seconds. No significant differences could be identified between variants, temperatures, or time points.

pH measurement after reconstitution:

pH values fluctuated only slightly around the target pH of 7.3 (final formulation variants #1 and #1d) and 8.1 (final formulation variants #2 and #2d), respectively, by up to 0.1 pH units. This is within the expected range and no significant differences could be identified between variants, temperatures, or time points.

Residual water content using a generic Karl-Fischer oven method (in duplicate):

Residual water content was identified as 0.2% to 0.5% right after lyophilization across all variants. During storage the values increased slightly in all variants to about 0.5 % to 0.6 % at 25 °C storage temperature and to about 0.7 % at 40 °C storage temperature. The accumulation of water in the lyophilizates seems to be more or less finished after 8 weeks of storage at both temperatures as no further increase could be detected after 12 weeks. No significant differences could be identified between formulation variants.

Aggregation status by SE-HPLC:

Right after lyophilization samples showed an increased aggregate/fragment content compared to the reference drug substance by up to 1.5 percentage points (final formulation variant #2d). After 8 weeks of storage all samples were comparable with relative main peak areas of around 99.0%. After 12 weeks all variants were still comparable but showed a lower aggregates/fragments

content (merely 0.5%) than after 8 weeks. This could be due to aggregates/fragments being further degraded to species that are not detectable by the method anymore.

Chemical degradation by RP-HPLC:

Right after lyophilization, all variants showed a GM-CSF purity of 97.7% - 98.0%. The purity in the reference drug substance was identified as 98.8%. Storage for 8 weeks led to a decrease in purity by around 0.6 percentage points in all variants at 25°C, whereas samples stored at 40°C showed a reduction in purity by around 1.0%. After 12 weeks samples stored at 25°C showed around 97% purity, whereas samples stored at 40 °C showed 96.1% to 96.4% of purity. No significant differences could be identified between formulation variants.

Nano-particle analysis by DLS:

Most samples showed more than 98% of mass fraction in the expected range for GM-CSF of 0.1-10 nm. As it appears, some samples showed lower mass fractions in that range by up to 3 percentage points rather randomly. No pattern indicating accumulation of larger molecules at a specific temperature or after a specific time could be identified..

Overall, all final formulation variants seem equally feasible for a GM-CSF lyophilized drug product.

Example 9

1. Scope and Objective

This study was aimed to determine any differences in the potential local tolerance effect at the site of injection of formulations of the invention and an existing commercial formulation of GM-CSF following repeated intradermal administration to rabbits. Two variants of formulations of the invention were evaluated, namely, final formulation variant #1 and final formulation variant #2. The study was completed while adhering to GLP requirements.

2. Methods and Apparatus

2.1 Preparation of the Test Items and Vehicle

Table 59 – Test Items and Vehicle

Test Item 1	Final formulation variant #1
Test Item 2	Final formulation variant #2
Test Item 3	Existing commercial formulation
Vehicle	Final formulation variant #2 without GM-CSF

The test items and vehicle were reconstituted by adding water for injection (WFI; aqua ad inject.) as follows:

- The content of one vial of the vehicle (0.1 mg) was dissolved in 330 µL of WFI. The solution was stored at room temperature and used within 6 hours after reconstitution.
- The content of one vial of the test item 1, Final formulation variant # 1, (0.1 mg) was dissolved in 330 µL of WFI. The solution was stored at room temperature and used within 6 hours after reconstitution.
- The content of one vial of the test item 2, Final formulation variant # 2, (0.1 mg) was dissolved in 330 µL of WFI. The solution was stored at room temperature and used within 6 hours after reconstitution.
- The content of one vial of the test item 3, an existing commercial formulation, (0.1 mg) was dissolved in 330 µL of WFI. The solution was stored at room temperature and used within 6 hours after reconstitution.

For solution of the 3 test items and the vehicle in WFI, the mixtures were swirled gently for 2 minutes by hand and then allowed to stand at room temperature for 11 minutes until the lyophilized cake was reconstituted to a clear solution. The formulations were prepared freshly on each administration day before the administration.

2.2 Test Systems

Species/strain: healthy New Zealand White Rabbits, Crl: KBL (NZW)
Source: Charles River Deutschland, 97633 Sulzfeld, Germany
Sex: male
Age at the start of the acclimatisation period: approximately 12 weeks
Body weight at the start of the acclimatisation period: approximately ≥ 1.8 kg
Body weight at the start of the treatment period: 2.3–3.0 kg
Number of animals: 4 animals / main study
4 animals / recovery group

2.2.1 Housing and Feeding Conditions

- Semi barrier in an air-conditioned room
- Temperature: 18 ± 3 °C
- Relative humidity: 55 ± 10%
- Artificial light, sequence being 12 hours light, 12 hours dark
- Air change: 10 x / hour

- Free access to irradiated hay briquettes or autoclaved hay and to Altromin 2123 maintenance diet for rabbits, rich in crude fibre
- Free access to tap water (drinking water, municipal residue control, microbiological controls at regular intervals)
- Certificates of food, water and bedding are filed for two years at BSL Munich and afterwards archived at Eurofins Munich
- Housed in ABS-plastic or Noryl rabbit cages, floor 4200 cm^2
- Adequate acclimatisation period (at least 5 days) under laboratory conditions

2.3 Groups and Identification of the Animals

Each animal was marked for individual identification by a tattoo number and an additional ink marked number in the ear.

The study was conducted with two groups (main study and recovery group) containing four animals (Table 60).

Table 60: Animal Identification

Group Number	Group	Number/Sex of Animals	Animal Number	Time of Necropsy
1	main study	4/male	1-4	day 10
2	recovery	4/male	5-8	day 24

2.4 Experimental Procedure

Approximately 8 hours before administration on days 1, 3 and 5, and approximately 5 hours before administration on day 8, the hair at the injection sites was carefully removed. Care was taken to avoid mechanical irritation and trauma.

Each animal received single intradermal injections at four injection sites: test item 1, test item 2, test item 3 and the vehicle were administered on day 1, day 3, day 5 and day 8 of the study. The injection sites were placed about 2.5 cm apart from the midline and about 5 cm apart from each other. Two injection sites were on the left hand side of the animal and two injection sites were on the right hand side of the animal, with two sites towards the head and two sites towards the caudal region. Each substance was assigned to one of four injection sites (Table 61) and was always administered at the same injection site in each animal. The positions of the injection sites are shown in Table 62. The exact injection sites were marked with a coloured pen prior to dosing and thereafter. The administration volume at each Injection site was 0.1 mL.

Towards the caudal region, an untreated skin site was sampled as a control, on the left and right side of the back of each animal at necropsy, which was not treated with test item 1, 2 and 3 or the vehicle. The samples were investigated histopathologically and described as sites 5 and 6 herein.

Table 61: Assignment and Administration of the Substances

Injection Site	Substance	Route of Administration	Volume of Administration
1	vehicle	intradermal	0.1 mL
2	Existing commercial formulation		
3	Final formulation variant # 1		
4	Final formulation variant # 2		

The vehicle, the test item 1, test item 2 and test item 3 were administered as follows:

Injection site 1: 0.1 mL vehicle

Injection site 2: 0.1 mL existing commercial formulation

Injection site 3: 0.1 mL final formulation variant # 1

Injection site 4: 0.1 mL final formulation variant # 2

Table 62: Position of the Injection Sites

	Animal 1	Animal 2	Animal 3	Animal 4
Group 1 (main study)	Head ↑ 1 2 / 4 3	Head ↑ 4 1 / 3 2	Head ↑ 3 4 / 2 1	Head ↑ 2 3 / 1 4
	Animal 5	Animal 6	Animal 7	Animal 8
Group 2 (recovery group)	Head ↑ 1 2 / 4 3	Head ↑ 4 1 / 3 2	Head ↑ 3 4 / 2 1	Head ↑ 2 3 / 1 4

1-4 = injection site 1-4

The animals of the main study were observed for a period of 10 days. The animals of the recovery group were observed for a period of 24 days.

2.5 Clinical Observations

The general health of the animals was checked once daily during the observation and recovery period. The skin was examined to evaluate the degree of erythema, oedema, desquamation, scab formation and any other lesions 1 hour after the injections on day 1, day 3, day 5 and day 8, and once daily on each of the non-dosing days for the duration of the observation and recovery period. For animals no. 5 – 8 of the recovery group the clinical observation and evaluation of the scoring according to Table 63 was not performed on study day 17. All animals in the study are checked for mortality on this day. The tissue reaction, erythema and oedema, was graded for all the injection sites according to 79. Any other signs or systemic effects were recorded.

Table 63: Classification System (Erythema/Oedema)

Erythema and Eschar Formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4
Oedema Formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond exposure area)	4

2.6 Body Weight Development

The animals were weighed at least once weekly from day 1 on and on the day of necropsy, i.e. on day 10 (animals of the main study) or day 24 (animals of the recovery group).

2.7 Evaluation of Results

The individual scores for each animal were recorded according to the scoring system described in Table 63.

2.8 Pathology

At the end of the observation or recovery period, the animals were euthanised with an overdose of anaesthetic. The animals of the main study were euthanised on study day 10 and the animals of the recovery group on study day 24.

After their examination and macroscopic evaluation, all injection sites (injection site 1, 2, 3 and 4), together with sufficient unaffected tissue around each injection site and any macroscopic abnormalities were excised to enable the evaluation of the biological response. Untreated skin (i.e. not treated with test item 1, 2 and 3 or the vehicle) from the region caudal from the injection sites, on the left and right side of the back of each animal, was sampled as a negative control (untreated control sites) at necropsy. The samples were investigated histopathologically and described as sites 5 and 6 herein. The tissue samples from the injection sites as well as the negative controls were fixed in a 10% neutral buffered formalin solution.

2.9 Histopathology

Histological processing of all preserved tissues to haematoxylin and eosin stained microscope slides (approximate thickness of 4 µm) was performed according to Good Laboratory Practices. The samples were trimmed, processed, embedded in paraffin wax and cut at an approximate thickness of 2-4 micrometers, and stained with hematoxylin and eosin. The slides were checked under the microscope for quality and evaluated by light microscope.

A scoring system, as proposed by ISO 10993-6:2016(E) was applied (Table 64).

Table 64: Score System Adapted to ISO 10993-6:2016(E)

Cell type/response	Score				
	0	1	2	3	4
Polymorphonuclear cells	0	Rare,1-5/hpf	5-10/hpf	Heavy infiltrate	Packed
Lymphocytes	0	Rare,1-5/hpf	5-10/hpf	Heavy infiltrate	Packed
Plasma cells	0	Rare,1-5/hpf	5-10/hpf	Heavy infiltrate	Packed
Macrophages	0	Rare,1-5/hpf	5-10/hpf	Heavy infiltrate	Packed
Giant cells	0	Rare,1-2/hpf	3-5/hpf	Heavy infiltrate	Sheets
Necrosis	0	Minimal	Mild	Moderate	Severe
Edema	0	Minimal	Mild	Moderate	Severe
Calculation	Subtotal (x2)				
Neovascularisation	0	Minimal capillary proliferation,	Groups of 4-7 capillaries with supporting	Broad band of capillaries with supporting	Extensive band if capillaries with supporting

		focal, 1-3 buds	fibroblastic structures	structures	fibroblastic structures
Fibrosis	0	Narrow band	Moderately thick band	Thick band	Extensive band
Fatty Infiltrate	0	Minimal amount off at associated with fibrosis	Several layers of fat and fibrosis	Elongated and broad accumulation of fat calls about the implant site	Extensive fat completely surrounding the implant
Calculation	Subtotal				
	Total				
	Group Total				
	Average				

Furthermore, the type of inflammation was characterized according to its nature and the severity was graded on a scale of 1 to 5 (minimal to severe; Table 65).

Table 65: grading of type of inflammation

Grade	Severity	Nature
Grade 1	Minimal	Histopathologic change ranging from inconspicuous to barely noticeable but so minor, small, or infrequent as to warrant no more than the least assignable grade. For multifocal or diffusely-distributed lesions, this grade was used for processes where less than approximately 10% of the tissue in an average high-power field was involved.
Grade 2	Slight	Histopathologic change that is a noticeable but not a prominent feature of the tissue. For multifocal or diffusely-distributed lesions, this grade was used for processes where between approximately 10% and 25% of the tissue in an average high-power field was involved.
Grade 3	Moderate	Histopathologic change that is a prominent but not a dominant feature of the tissue. For multifocal or diffusely-distributed lesions, this grade was used for processes where between approximately 25% and 50% of the tissue in an average high-power field was involved.
Grade 4	Marked	Histopathologic change that is a dominant but not an overwhelming feature of the tissue. For multifocal or diffusely-distributed lesions, this grade was used for processes where between approximately 50% and 95% of the tissue in an average high-power field was involved.
Grade	Severe	Histopathologic change that is an overwhelming feature of the tissue. For

5		multifocal or diffusely-distributed lesions, this grade was used for processes where greater than approximately 95% of the tissue in an average high-power field was involved.

3. Results

The administration of final formulation variant # 1, final formulation variant # 2, an existing commercial formulation or vehicle as single intradermal injections on four occasions to male rabbits was not associated with any signs of systemic toxicity in any animal. There were no unscheduled deaths during this study.

3.1 Clinical Signs and Local Effects at the Application Site

No mortalities and no signs of systemic toxicity were recorded during the observation period for any of the animals. Three animals of Group 2 (nos. 5, 6 and 7) showed slight diarrhoea on day 8 and day 9. Upon consideration of the absence of other clinical findings, the diarrhoea was deemed to be incidental. There were no further clinical findings recorded during the observation period for any of the animals.

There were no local effects associated with vehicle administration at injection site 1.

Local effects were mainly recorded at injection site 2, treated with an existing commercial formulation (test item 3), for all animals in each group. Erythema up to grade 3 (moderate to severe) and oedema up to grade 4 (severe) were found at injection site 2.

At injection site 3 (final formulation variant # 1), erythema grade 1 (very slight) was recorded for animal no.4 on days 9 and 10 and animal no. 6 on days 10 and 11.

Final formulation variant # 2 administered to injection site 4 was associated with erythema grade 2 (well defined) for animal no. 3 on days 9 and 10, and erythema grade 1 on day 2 for animal no. 5. A small firm lump was noted on day 10 at injection site 4 for animal no. 7. As this finding was seen on one single observation day in one animal this finding was considered to be incidental.

In the animals of Group 1, the findings persisted until the end of the observation period of 10 days, whereas in the animals of Group 2, the findings were completely reversed before the end of the observation period of 24 days.

For details, see Tables 66 and 67.

Table 66: Clinical Signs and Local Effects– Group 1

Clinical Signs – Group 1					
Animal No. 1					
Time of Observation	Clinical Signs	Local Effects at Injection Site			
		1	2	3	4
Day 1 – Day 8	nsf	nsf			
Day 9 – Day 10	nsf	nsf	E3, O4	nsf	nsf
Animal No. 2					
Time of Observation	Clinical Signs	Local Effects at Injection Site			
		1	2	3	4
Day 1 – Day 7	nsf	nsf			
Day 8	nsf	nsf	E1	nsf	nsf
Day 9 – Day 10	nsf	nsf	E2, O4	nsf	nsf
Animal No. 3					
Time of Observation	Clinical Signs	Local Effects at Injection Site			
		1	2	3	4
Day 1 – Day 8	nsf	nsf			
Day 9	nsf	nsf	E2, O4	nsf	E2
Day 10	nsf	nsf	E2, O3	nsf	E2
Animal No. 4					
Time of Observation	Clinical Signs	Local Effects at Injection Site			
		1	2	3	4
Day 1 – Day 7	nsf	nsf			
Day 8	nsf	nsf	E1	nsf	nsf
Day 9	nsf	nsf	E2, O4	E1	nsf
Day 10	nsf	nsf	E2, O3	E1	nsf

nsf = no specific findings; E(1-4) = erythema (grade 1-4); O(1-4) = oedema (1-4)

Table 67: Clinical Signs and Local Effects– Group 2

Clinical Signs – Group 2					
Animal No. 5					
Time of Observation	Clinical Signs	Local Effects at Injection Site			
		1	2	3	4
Day 1	nsf	nsf			
Day 2	nsf	nsf	E1	nsf	E1

Day 3 – Day 7	nsf	nsf			
Day 8	slight diarrhoea	nsf	E1	nsf	nsf
Day 9	slight diarrhoea	nsf	E3, O4	nsf	nsf
Day 10	nsf	nsf	E3, O4	nsf	nsf
Day 11	nsf	nsf	E2, O3	nsf	nsf
Day 12	nsf	nsf	E2, O2	nsf	nsf
Day 13 – Day 16	nsf	nsf	E2, O1	nsf	nsf
Day 17	no evaluation of data				
Day 18	nsf	nsf	E2, O1	nsf	nsf
Day 19	nsf	nsf	E2	nsf	nsf
Day 20 – Day 24	nsf	nsf			
Animal No. 6					
Time of Observation	Clinical Signs	Local Effects at Injection Site			
		1	2	3	4
Day 1 – Day 7	nsf	nsf			
Day 8	slight diarrhoea	nsf			
Day 9	slight diarrhoea	nsf	E2, O4	nsf	nsf
Day 10 - Day 11	nsf	nsf	E2, O4	E1	nsf
Day 12	nsf	nsf	E2, O3	nsf	nsf
Day 13 – Day 16	nsf	nsf	E2, O2	nsf	nsf
Day 17	no evaluation of data				
Day 18	nsf	nsf	E2, O2	nsf	nsf
Day 19 – Day 20	nsf	nsf	E1	nsf	nsf
Day 21 – Day 24	nsf	nsf			
Animal No. 7					
Time of Observation	Clinical Signs	Local Effects at Injection Site			
		1	2	3	4
Day 1 – Day 7	nsf	nsf			
Day 8	slight diarrhoea	nsf			
Day 9	slight diarrhoea	nsf	E2, O4	nsf	nsf

Day 10	nsf	nsf	O2	nsf	sN
Day 11 - Day 12	nsf	nsf	O1	nsf	nsf
Day 13 – Day 16	nsf	nsf			
Day 17	no evaluation of data				
Day 18 – Day 24	nsf	nsf			

nsf = no specific findings;E(1-4) = erythema (grade 1-4); O(1-4) = oedema (1-4);
sN = small, firm lump in skin

3.2 Body Weight Development

All animals showed weight gain on the last observation day compared with the day of application, and no relevant weight loss was observed during the study period (see Tables 68 and 69).

Table 68: Absolute Body Weights – Group 1

Body Weights [kg] – Group 1				
Animal No.	1	2	3	4
Day 1	2.6	2.5	2.9	2.3
Day 8	2.8	2.6	3.0	2.4
Day 10 (day of necropsy)	2.9	2.6	3.0	2.5

Table 69: Absolute Body Weights – Group 2

Body Weights [kg] – Group 2				
Animal No.	5	6	7	8
Day 1	2.8	3.0	2.4	2.3
Day 8	2.7	3.3	2.6	2.4
Day 15	2.8	3.3	2.7	2.5
Day 22	2.8	3.3	2.8	2.7
Day 24 (day of necropsy)	2.9	3.4	2.9	2.8

3.3 Histopathology - Macroscopic Findings

The macroscopic evaluation of the injection sites as well as the untreated skin was conducted according to the classification system described in Table 63. No findings were recorded at any of the injection sites. For animal nos. 3 and 4, oedema was seen near the injection site 2 (an existing commercial formulation). For individual data see Tables 70 and 71.

Table 70: Macroscopic Findings – Group 1

Macroscopic Findings – Group 1					
Animal No.	**Injection Site**				
	1 vehicle	2 (Existing commercial formulation)	3 (Final formulation variant # 1)	4 (Final formulation variant # 2)	Untreated Skin (Control)
	E/O	E/O	E/O	E/O	E/O
1	0/0	0/0	0/0	0/0	0/0
2	0/0	0/0	0/0	0/0	0/0
3	0/0	0/0*	0/0	0/0	0/0
4	0/0	0/0*	0/0	0/0	0/0

E = erythema; O = oedema; 0-4 = grade erythema/oedema; *oedema adjacent to injection site

Table 71: Macroscopic Findings – Group 2

Macroscopic Findings – Group 2					
Animal No.	**Injection Site**				
	1 vehicle	2 (Existing commercial formulation)	3 (Final formulation variant # 1)	4 (Final formulation variant # 2)	Untreated Skin (Control)
	E/O	E/O	E/O	E/O	E/O
5	0/0	0/0	0/0	0/0	0/0
6	0/0	0/0	0/0	0/0	0/0
7	0/0	0/0	0/0	0/0	0/0
8	0/0	0/0	0/0	0/0	0/0

E = erythema; O = oedema; 0-4 = grade erythema/oedema

3.4 Histopathology - Microscopic Findings

3.4.1 Main Group

There were no significant differences between vehicle, final formulation variant # 1 and final formulation variant # 2. The scores at these injection sites were slightly higher than the scores of

untreated skin sites. The latter were affected in some animals by inflammatory lesions that were deemed to have a close anatomical relationship to injection sites (Figures 48 and 49,). The highest inflammation score was however noted at the injection sites of the existing commercial formulation (Table 72, Figures 46 and 47).

Table 72: Summarised scores of the main group

	Vehicle	**Existing commercial formulation**	**Final formulation variant # 1**	**Final formulation variant # 2**	**Untreated control sites**
Group Total	37	100	30	32	46
Sample number	4	4	4	4	8
Average	9.3	25	7.5	8	5.8

The inflammatory lesions could be summarized as inflammation that was either subacute or necrotizing (Table 73). Again, the existing commercial formulation was the most heavily affected by necrotizing inflammation. However, necrotizing inflammation was also noted at other injection sites, e.g., in animal no. 4, there was necrotizing inflammation at the vehicle site.

Table 73: Inflammation recorded at skin sites

Animal no.	**Injection site**	**Inflammation Type**
1	Existing commercial formulation	Inflammation, necrotizing, grade 3
	Final formulation variant # 1	Inflammation, subacute, grade 1
	Final formulation variant # 2	Inflammation, subacute, grade 1
2	Vehicle	Inflammation, subacute, grade 1
	Existing commercial formulation	Inflammation, necrotizing, grade 4
	Untreated control site	Inflammation, subacute, grade 2
3	Existing commercial formulation	Inflammation, necrotizing, grade 3
	Final formulation variant # 1	Inflammation, necrotizing, grade 2
	Final formulation variant # 2	Inflammation, necrotizing, grade 2
	Untreated control site	Inflammation, necrotizing, grade 3
4	Vehicle	Inflammation, necrotizing, grade 4
	Existing commercial formulation	Inflammation, necrotizing, grade 3
	Final formulation variant # 1	Inflammation, necrotizing, grade 3
	Final formulation variant # 2	Inflammation, necrotizing, grade 2

3.4.2 Recovery Group

The highest inflammation score was noted at the injection sites of the existing commercial formulation and final formulation variant #1. There was minimal inflammation noted at vehicle and final formulation variant #2 injection sites. The lowest inflammation score was noted in untreated control sites (Table 74, Figures 50-53).

Table 74: Summarised scores of the recovery group

	Vehicle	**Existing commercial formulation**	**Final formulation variant # 1**	**Final formulation variant # 2**	**Untreated control sites**
Group Total	6	17	18	4	2
Sample number	4	4	4	4	8
Average	1.5	4.3	4.5	1	0.3

The inflammatory scores can be interpreted as being chronic inflammation. The chronic inflammation was minimal and was only recorded at a total of three injection sites (the existing commercial formulation, final formulation variant # 1, and final formulation variant # 2) (Table 75).

Table 75: Inflammation recorded at skin sites

Animal no.	**Injection site**	**Inflammation Type**
5	Existing commercial formulation	Inflammation, chronic, grade 1
	Final formulation # 1	Inflammation, chronic, grade 1
6	Final formulation variant # 2	Inflammation, chronic, grade 1

4. Conclusions

From the findings recorded histologically, there were inflammatory lesions at all injection sites but also at untreated control sites.

In animals sacrificed after 10 days, the highest inflammatory score was noted at the existing commercial formulation injection sites. There were no significant difference between the vehicle, final formulation variant #1 and final formulation variant #2, but all three had a higher score than untreated control sites. A relatively high inflammatory score was noted in the untreated skin sites, which was considered to be due to extending inflammatory lesions from injection sites. The inflammatory lesions can be described as subacute or necrotizing inflammation. The existing

commercial formulation was most heavily affected by necrotizing inflammation. However, necrotizing inflammation was also noted at other injection sites.

After 24 days, inflammatory scores were in general low. The highest inflammation score was noted at the injection sites of the existing commercial formulation and final formulation variant #1. There was less inflammation noted at the vehicle and final formulation variant #2 injection sites. The lowest inflammation score was noted in untreated control sites. The inflammatory scores can be interpreted as being chronic inflammation. The chronic inflammation was minimal and was only recorded at a total of three injection sites (existing commercial formulation, final formulation variant #1, and final formulation variant #2).

To summarise, final formulation variant #1 and final formulation variant #2 were deemed to be less of an irritant than the existing commercial formulation. However, the finding from the recovery group showed that final formulation #2 had the lowest inflammatory score.

Example 10

1. Aim of the Study

The objective of this study was to determine relative potency of 14 GM-CSF samples from Example 2 by the TF-1 cell proliferation assay setup according to monograph European Pharmacopoeia 01/2005:1641. The potency assay is a proliferation bioassay based upon the ability of GM-CSF to provoke the proliferation of the TF-1 erythroblast cell line. In this study, the potency of 14 GM-CSF formulations was measured.

2. Test and Reference Items Information

2.1 Test Items

Table 76: Test items (samples)

Name	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**
Test Item No.	**1**	**2**	**4**	**5**	**6**	**7**	**8**
Lot No.	150904/ 1064/71	161103/ 1150/157	TG WP3 FD				
Description	Control Sample 1	Control Sample 2	Sub-variant #1 P188	Sub-variant #1 P188 air	Sub-variant #3 P188 air	Sub-variant #3 P188 air	Sub-variant #3 P188 N2

			air T0	40°C	T0	40°C	40°C
Quantity	2 x 1ml	2 x 1ml	n/a	n/a	n/a	n/a	n/a
Protein concentration mg/ml	3.46	4.8	0.3	0.3	0.3	0.3	0.3
Storage condition	-80±5°C						

Table 76 continued:

Name	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**	**rHu-GM-CSF**
Test Item No.	**9**	**10**	**11**	**12**	**13**	**3**	**14**
Lot No.	TG WP3 FD					170118/ 1167/095	201602A01
Description	Sub-variant #4 P188 N2 40°C	Sub-variant #3 w/o air T0	Sub-variant #3 w/o air 40°C	Sub-variant #3 rHA air 40°C	Sub-variant #4 w/o air 40°C	Control Sample 3	Molgramostim (existing commercial formulation)
Quantity	n/a	n/a	n/a	n/a	n/a	1 ml/vial	100 µg/vial
Protein concentration mg/ml	0.3	0.3	0.3	0.3	0.3	5.0	n/a
Storage condition	-80±5°C						5±3°C

2.2 Reference item

Table 77: Reference item

Reference item ID	WHO International Standard Granulocyte Macrophage Colony Stimulating Factor (here referred to as: WHO GM-CSF Standard)
Storage temperature stock solution	-80±5°C
Protein concentration	1 µg/ml

Activity	10,000 International Units/ml.
Supplier/distributor	NIBSC

3. Test System

Table 78: Test system

Test system	**Supplier**	**Cat. No.**	**Lot No. /Serial No.**
TF-1 cell line (working stock)	IBR Inc.	ATCC, CRL-2003	Passage 4
Microplate Reader MRX with Revelation Version 4.25	Dynatec	n/a	2CXA1186

4. Justification of Test System

The unknown stimulatory activity of GM-CSF formulations is compared to a reference standard in order to quantify the potency of the GM-CSF formulations. As a Standard, the WHO international Standard GM-CSF is used as the reference standard. The test system is the TF-1 cell proliferation assay basing on Monograph EMA (01/2005:1641, Page 2054-2057, Molgramostim concentrated solution), the minimum content of GM-CSF (*Molgramostim)* is 2.0 mg of protein/ml with a minimum potency of 0.7×10^7 IU/mg of protein.

5. Analytical Methodology

5.1 Cultivation of TF-1 cells

5.1.1 Cultivation procedure

TF-1 cells were thawed and cultivated at 37°C/5% CO_2 in RPMI1640 medium supplemented with 10 % FBS and 2 ng/ml recombinant human GM-CSF. TF-1 cultures were seeded at a concentration approximately 1 x 10^5 of viable cells/ml. In 2 to 3 day intervals, the growth medium was either replaced with growth medium or, if the cell concentration exceeded 5 x 10^5 of viable cells/ml, the cells were split.

5.1.2 Experimental set-up

In order to obtain a final density of $1.5x10^4$ cells/well in the planned TF-1 cell proliferation assay, an adequate number of TF-1 cells, adjusted to 2-3 x 10^5 viable cells/ml, was suspended in GM-CSF-free Starving Medium and incubated at 37°C/5% CO_2 for 24±1 h. During starving in absence of GM-CSF, the TF-1 cells stop proliferation. After starvation, the cells were harvested and adjusted to 3 x 10^5 cells/ml in Starving Medium. A volume of 50 µl/well of the cell suspension was transferred to a flat bottom 96 well plate.

Table 79: Materials and reagents

Material	Supplier	Cat. No.
RPMI-1640	Sigma	R8758
FBS	Hyclone	SH30080.03H
GM-CSF for growth medium	Peprotec	300-03
Cell culture flask	Becton Dickenson	353024
WHO GM-CSF Standard	NIBSC	88/646
Trypan Blue	Gibco BRL	T15250-061

Table 80: TF-1 growth medium

Reagent	Final Concentration	Volume
RPMI1640	90 %	180 ml
FBS	10%	20 ml
GM-CSF (stock: 1 µM, corresponding to 14,6 µg/ml)	2 ng/ml	27.3 µl
Gentamycin (Stock 50 mg/ml)	20 µg /ml	80 µl

Table 811: TF-1 Starving medium

Reagent	Final Concentration	Volume
RPMI1640	90 %	180 ml
FBS	10%	20 ml
Gentamycin (Stock 50 mg/ml)	20 µg /ml	80 µl

5.2 Sample preparation

The Test Items (14 samples) were delivered as a stock solution in different formulation buffers with a pH ranging from 7.3 - 8.1, except the Test Item 14 which was delivered as lyophilisate. The lyophilisate was resuspended in PBS, pH 7.4 to a stock concentration of 0.1 mg/ml. All other Test Items were pre-diluted in PBS, pH 7.4 to the same stock concentration of 0.1 mg/ml.

Each Test Item was further diluted in RPMI1640/10% FBS to reach a unique concentration of 12ng/ml. Subsequently, for each of the 14 Test Items and the reference item WHO Standard, a 2x series of 11 concentrations starting at 12ng/ml was prepared in Starving Medium. The 2x series of the 11 concentrations were finally 1:2 diluted in the TF-1 cell proliferation assay.

In addition, the pH of each Test Item, including the reference item WHO Standard, was determined in a separate aliquot at its highest final concentration of 6ng/ml applied in the TF-1 cell proliferation assay. All dilutions were prepared in RPMI1640/10% FBS.

5.3 TF-1 proliferation assay

5.3.1 Procedure

The starved TF-1 cells were adjusted to 3 x 10^5 cells/ml and a volume of 50µl/well was pipetted into a flat bottom 96-well plate. Subsequently, 50µl/well of the 2x series of 11 concentrations of the 14 test items and the reference item WHO Standard were added to the TF-1 cells to obtain a final assay volume of 100µl. The final GM-CSF concentrations are described in Table 82. The dilution series of the WHO Standard were part of each assay plate.

Plate 1: WHO Standard and Control Sample 1, 12 concentrations and 11 concentrations, respectively, quadruplicate determinations in Starving Medium.

Plate 2-14: WHO Standard and Test Items, 12 concentrations and 11 concentrations, respectively, quadruplicate determinations in Starving Medium.

The plates were incubated at 37°C/5% CO_2 for 24 h and prior to cell proliferation determination by WST-1 reagent.

5.3.2 Proliferation measurement

The 24h proliferation response of TF-1 cells to the GM-CSF concentrations listed in Table 82 was determined in quadruplicate after addition of WST-1 reagent (10 µl/well), and the OD values were measured at 450 nm by the Microplate Reader MRX after incubation at 37°C for 180 min.

Table 82: GM-CSF concentrations for TF-1 cell qualification

Dilution No.	**Test and Reference Items Assay final concentrations**
	[ng/ml]
1	6.0000
2	2.0000
3	0.6667
4	0.2222

5	0.0741
6	0.0247
7	0.0082
8	0.0027
9	0.0009
10	0.0003
11	0.0001
12	0.0000

5.3.3 Data evaluation

The Mean OD and the Standard deviation (SD) of the quadruplicates were calculated by the Excel software. The Precision of the quadruplicate measurements was calculated by the following formula:

CV= SDx100/Mean.

The acceptance criterion for intra-assay (replicates on plate) precision was CV ≤ 20%. The proliferation rate was calculated by normalizing the OD of the various samples against the 0 ng/ml GM-CSF sample.

Proliferation rate= Mean OD at x ng/ml GM-CSF / Mean OD at 0 ng/ml

The dose response data of the WHO GM-CSF Standard and Test GM-CSF subsets was fitted by the XLfit software Version 5.3.1 using the MMF equation and a 90 % confidence interval. The MMF equation calculates the A, B, C and D parameter (Table 83).

Table 83: MMF Equation Parameters

Parameters	Description
A	Maximum y value.
B	EC_{50} factor.
C	Minimum y value.
D	Slope factor.

The EC_{50} value was calculated using the following formula:

$EC_{50}= B^{1/D}$

The relative activity (%) of the test item was calculated based on the EC_{50} of WHO GM-CSF Standard (100%):

Relative activity (%) = EC_{50} WHO GM-CSF Standard * 100/ EC_{50} test item.

Based on the relative activity, the IU/µg Test Items was calculated:

Test Item GM-CSF IU/µg = Relative activity (%) *10000 IU/µg[1]/100

[1] concentration of WHO GM-CSF Standard

6. Result and Discussion

6.1 Inter-assay variance of EC_{50} of the WHO GM-CSF Standard in the TF-1 cell proliferation assay (Test Items 1 – 14)

Table 84: Inter-assay variance of EC_{50} of the WHO GM-CSF Standard in the TF-1 cell proliferation assay (Test Item 1 – 14)

Test Item	**EC_{50}**
	ng/ml
1	0.010
2	0.009
3	0.009
4	0.008
5	0.008
6	0.009
7	0.008
8	0.011
9	0.010
10	0.010
11	0.009
12	0.008
13	0.009
14	0.009
Mean	0.0091
SD	0.0009
%CV	9.7396

6.2 GM-CSF potency in 14 GM-CSF samples

Two of the 14 tested GM-CSF Test Items (Test Items 12 and 13) did not pass the acceptance criterion for minimal potency of human GM-CSF which is, according to the monograph Pharm. Eur. 01/2005:1641, ≥0.7 x 10^7 IU/mg (Table 85). It should be noted that Test Items 12 and 13 were included as comparative samples, for reference.

With a CV of 9.7396%, the inter-assay variance of the EC_{50} of the WHO GM-CSF Standard in the TF-1 cell proliferation assay, Test Items 1-14 passed the acceptance criterion %CV ≤20% (Table 84).

Table 85: Summary of GM-CSF potency in 14 GM-CSF samples

Test Item No.	**EC_{50} [IU/µg]**	**EC_{50} [IU/mg]**	**Specification ≥0.7 x 10^7 IU/mg**
1	7021.01	0.70 x 10^7	Passed
2	7146.55	0.71 x 10^7	Passed
3	7149.42	0.71 x 10^7	Passed
4	10023.44	1.00 x 10^7	Passed
5	9415.94	0.94 x 10^7	Passed
6	10976.97	1.09 x 10^7	Passed
7	10085.86	1.08 x 10^7	Passed
8	10363.91	1.03 x 10^7	Passed
9	9574.76	0.95 x 10^7	Passed
10	7958.67	0.79 x 10^7	Passed
11	7625.50	0.76 x 10^7	Passed
12	6738.55	0.67 x 10^7	Not passed
13	6089.89	0.60 x 10^7	Not passed
14	10368.11	1.03 x 10^7	Passed

Determination of pH of each Test Item showed pH values in the range from 7.81 -7.90 (data not shown). There is no evidence that these pH values interfered with the performance of the TF-1 cell proliferation assay.

Example 11

1. Aim of the Study

This study aimed to determine the relative potency of six formulations of GM-CSF by the TF-1 cells proliferation assay setup according to monograph European Pharmacopoeia 01/2005:1641. The GM-CSF potency assay is a proliferation bioassay based upon the ability of GM-CSF to induce proliferation of the erythroblastic cell line TF-1.

2. Test and Reference Items Information

2.1 Test items

Table 86: Test Items (samples)

Description	Control Sample 1	Molgramostim rHuGM-CSF (existing commercial formulation)	Final Formulation variant #1
Lot No.	150904/1064/71	201703A05	TGWP7b/1-170814
Quantity	2 vials	1 ampoule	2 ampoules
Physical condition	liquid	lyophilisate	lyophilisate
Volume per sample	1ml	380µl	330µl
Concentration	3.5mg/ml	115µg/ampoule	100µg/ampoule
Reconstitution volume per ampoule	n/a	380µl	330µl
Reconstituted by	n/a	sterile ultrapure water	sterile ultrapure water
Storage condition	-80±5°C	5±3°C	5±3°C

Table 86 cont.

Description	Final formulation variant #2	Final formulation variant #1d	Final formulation variant #2d
Lot No.	TGWP7b/2-170814	TGWP7b/1d-170814	TGWP7b/2d-170814
Quantity	2 ampoules	2 ampoules	2 ampoules
Physical condition	lyophilisate	lyophilisate	lyophilisate
Volume per sample	330µl	330µl	330µl

Concentration	100µg/ampoule	100µg/ampoule	100µg/ampoule
Reconstitution volume per ampoule	330µl	330µl	330µl
Reconstituted by	sterile ultrapure water	sterile ultrapure water	sterile ultrapure water
Storage condition	5±3°C	5±3°C	5±3°C

2.2 Reference items

Table 87: Reference item

Reference item ID	WHO International Standard for Granulocyte Macrophage Colony Stimulating Factor
Lot no.	Not available from NIBSC
Storage temperature stock solution	-80±5°C
GM-CSF Protein	approximately 1 µg ampoule
Activity	10,000 International Units of biological activity per ampoule.
Supplier/distributor	NIBSC

3. Test System

Table 88: Test System

Test system	**Supplier**	**Cat. No.**	**Lot No. /Serial No.**
TF-1 cell line (working stock)	IBR Inc.	ATCC, CRL-2003	Passage 4 thawed
Microplate Reader MRX with Revelation Version 4.25	Dynatec	n/a	2CXA1186

4. Justification of Test System

This study describes the procedure of TF-1 proliferation in response to rHu-GM-CSF (Molgramostim) stimulation. The unknown stimulatory activity of rHu-GM-CSF test items are compared to the stimulatory activity of the reference standard in order to quantify the relative potency of the test items. The WHO international Standard GM-CSF is used as the reference standard.

Based on Monograph EMA (01/2005:1641, Page 2054-2057, Molgramostim concentrated solution), the minimum content of GM-CSF (Molgramostim) is 2.0 mg of protein/ml with the minimum potency of 0.7×10^7 IU/mg of protein.

5. Analytical Methodology

5.1 Cultivation of TF-1 cells

5.1.1 Cultivation procedure

TF-1 cells were thawed and cultivated at 37°C/5% CO_2 in RPMI1640 medium supplemented with 10 % FBS and 2 ng/ml recombinant human GM-CSF. TF-1 cultures were seeded at a density of 10^5 viable cells/ml. In 2 to 3 day intervals the growth medium was either replaced or, if the cell concentration exceeded 5 x 10^5 viable cells/ml, the cells were split.

5.1.2 Experimental setup

The TF-1 cells were washed with Starving Medium to remove the TF-1 Growth Medium and adjusted to $2x10^5$ – $3x10^5$ cells/ml viable cells. Because TF-1 cells do not proliferate in Starving Medium the number of cells was adjusted to 1.5 x 10^4 cells/well, the cell number required for the assay 24h later. The cells were starved at 37°C/5% CO_2 for 24±3h. Thereafter, the TF-1 cells were harvested and diluted to $3x10^5$ cells/ml with Starving Medium. 50µl of the cell suspension was transferred into each well of a 96-well flat bottom plate except 2 wells that were used as Blank for the WST-1 reaction (medium without cells).

Table 89: Materials and Reagents

Material	**Supplier**	**Cat. No.**
RPMI-1640	Sigma	R8758
FBS	HyClone	SH30080.03H
GM-CSF for growth medium	PeproTech	300-03
Gentamycin	Sigma-Aldrich	G1397
WHO GM-CSF Standard	NIBSC	88/646
Trypan Blue	Gibco BRL	T15250-061

Table 90: TF-1 Growth medium

Reagent	Final Concentration	Volume
RPMI1640	90 %	180 ml
FBS	10 %	20 ml
GM-CSF (stock: 1 µM, corresponding to 14,6 µg/ml)	2 ng/ml	27.3 µl
Gentamycin (Stock 50 mg/ml)	20 µg /ml	80 µl

Table 91: TF-1 Starving medium

Reagent	Final Concentration	Volume
RPMI1640	90 %	180 ml
FBS	10%	20 ml
Gentamycin (Stock 50 mg/ml)	20 µg /ml	80 µl

5.2 Sample preparation

Of the test items, 5 samples were delivered as lyophilisate. Control Sample 1 was delivered as a solution at a concentration of 3.5 mg/ml. The GM-CSF lyophilisates were dissolved at 0.3 mg/ml in sterile ultrapure water by gentle rotation of the vial (reconstitution volume see Table 86) followed by a rest of 10 minutes. Then, the test items were pre-diluted in PBS pH 7.4 to a concentration of 0.1 mg/ml.

For the test items and the WHO Reference Standard, further dilutions were prepared in RPMI1640/10 % FBS; (Starving medium) to obtain a top 2X assay concentration of 12 ng/ml from which a 2X series of 11 GM-CSF concentrations was prepared in Starving Medium. Finally, the 2X series of dilutions became 1:2 diluted in the TF-1 cell proliferation assay (for final concentrations see Table 92).

5.3 TF-1 proliferation assay

5.3.1 Procedure

The starved TF-1 cells were adjusted to $3x10^5$ cells/ml and the volume of 50 µl/well was pipetted into a flat bottom 96-well plate. Subsequently, 50 µl/well of the 2x series of 11 concentrations of the 6 Test Items and the WHO GM-CSF Reference Standard were added to the TF-1 cells. The final assay volume was 100 µl. The final GM-CSF concentrations are described in Table 92. The dilution series of the WHO Standard were part of each assay plate.

Plate 1: WHO Standard (reference item) and the Control Sample 1, 12 concentrations and 11 concentrations, respectively, quadruplicate determinations in Starving Medium.

Plate 2-6: WHO Standard (reference item) and Test Item samples, 12 concentrations and 11 concentrations, respectively, quadruplicate determinations in Starving Medium.

The plates were incubated at 37°C/5% CO_2 for 24 h prior to cell proliferation determination by WST-1 reagent.

5.3.2 Proliferation measurement

The 24h proliferation response of TF-1 cells to the rHu-GM-CSF concentrations (Table 92) was determined in quadruplicate with WST-1 reagent (10 µl/well). OD values were measured at 450 nm by the Microplate Reader MRX after incubation of the cells at 37°C for 180 min.

Table 92: GM-CSF concentrations for TF-1 cell qualification

Dilution No.	**Test and Reference Items Assay final concentrations**
	[ng/ml]
1	6.0000
2	2.0000
3	0.6667
4	0.2222
5	0.0741
6	0.0247
7	0.0082
8	0.0027
9	0.0009
10	0.0003
11	0.0001
12	0.0000

5.4 Data evaluation

The Mean OD and the Standard deviation (SD) of the quadruplicates were calculated by the Excel software. The Precision of the quadruplicate measurements was calculated by the following formula:

CV= SDx100/Mean.

The acceptance criterion for intra-assay (replicates on plate) precision was CV ≤ 20%.

The proliferation rate was calculated by normalizing the OD of the various samples against the 0 ng/ml GM-CSF sample.

Proliferation rate= Mean OD at x ng/ml GM-CSF / Mean OD at 0 ng/ml

The dose response data of the WHO GM-CSF Standard and Test GM-CSF subsets was fitted by the XLfit software Version 5.3.1 using the MMF equation and a 90 % confidence interval. The MMF equation calculates the A, B, C and D parameter (Table 93).

Table 93: MMF Equation Parameters

Parameters	Description
A	Maximum y value.
B	EC_{50} factor.
C	Minimum y value.
D	Slope factor.

The EC_{50} value was calculated using the following formula:

$EC_{50}= B^{1/D}$

The relative activity (%) of the test item was calculated based on the EC_{50} of WHO GM-CSF Standard (100%).

Relative activity (%) = EC_{50} WHO GM-CSF Standard x 100/ EC_{50} test item.

Based on the relative activity, the IU/µg test items was calculated.

Test Item GM-CSF IU/µg = Relative activity (%) x 10000 IU/µg[1]/100

[1] concentration of WHO GM-CSF Standard

6. Results and Discussion

6.1 Inter-assay variance of EC_{50} of the WHO GM-CSF Standard in the TF-1 cell proliferation assay (Test Item 1-6)

Table 94: Inter-assay variance of EC_{50} of the WHO GM-CSF Standard in the TF-1 cell proliferation assay (Test Item 1-6)

Plates ID	GM-CSF WHO Standard EC_{50}	Mean	SD	CV	Acceptance criterion
	[ng/ml]			[%]	CV ≤20%
A	0.011	0.013	0.001	10.66	Passed
B	0.013				
C	0.012				

D	0.015				
E	0.013				
F	0.014				

6.2 GM-CSF potency in 6 formulations

All of the 6 tested formulations passed the acceptance criterion for minimal potency of human GM-CSF which is, according to the monograph Pharm. Eur. 01/2005:1641, ≥0.7 x 10^7 IU/mg (Table 95). With a CV of 10.66%, the inter-assay variance of the EC_{50} of the WHO GM-CSF Standard in the TF-1 cell proliferation assay, Test Items 1-6 passed the acceptance criterion CV ≤20% (Table 95).

Table 95: Summary of GM-CSF potency in 6 formulations

Test Item No.	**EC_{50} [IU/µg]**	**EC_{50} [IU/mg]**	**Specification ≥0.7 x 10^7 IU/mg**
1	10475.31	1.05 x 10^7	Passed
2	12759.29	1.28 x 10^7	Passed
3	12037.15	1.20 x 10^7	Passed
4	13870.28	1.39 x 10^7	Passed
5	10601.55	1.06 x 10^7	Passed
6	11667.56	1.17 x 10^7	Passed

Example 12

1. Scope and Objective

As performed in Example 5 with final formulation variants containing sucrose, formulations containing sucrose and mannitol were lyophilized to determine the effect on the appearance of the lyo cake.

The following formulation variants were selected for this study.

Table 96 – Final formulation variants lyophilized in this study

Final Formulation Variant #	**Composition of liquid bulk drug substance**	**Composition of lyophilized drug product per vial**	**Fill volume per vial**
1-m	0.30 mg/mL GM-CSF 10 mM K-phosphate 58 mM sucrose 220 mM mannitol 0.1 % P188 pH 7.3	GM-CSF: 0.10 mg Phosphoric acid: 0.32 mg Sucrose: 6.6 mg Mannitol: 13.4 mg P188: 0.33 mg	0.33 mL

		Add KOH to pH 7.3	
2-m	0.3 mg/mL GM-CSF 10 mM K-phosphate 58 mM sucrose 220 mM mannitol 0.1 % P188 pH 8.1	GM-CSF: 0.10 mg Phosphoric acid: 0.32 mg Sucrose: 6.6 mg Mannitol: 13.4 mg P188: 0.33 mg Add. using KOH to pH 8.1	0.33 mL
1d-m	0.15 mg/mL GM-CSF 5 mM K-phosphate 29 mM sucrose 110 mM mannitol 0.05 % P188 pH 7.3	0.33 mL final formulation of #1-m + 0.33 mL WFI	0.66 mL
2d-m	0.15 mg/mL GM-CSF 5 mM K-phosphate 29 mM sucrose 110 mM mannitol 0.05 % P188 pH 8.1	0.33 mL final formulation of #2-m + 0.33 mL WFI	0.66 mL

The final formulation variants were compounded, sterile filtered through 0.1µm PES membrane filter and filled into cleaned and heat-sterilized 2R vials. Active vials (10 vials per final formulation variant) were partially stoppered with autoclaved and dried lyo stoppers. Active samples were loaded into a freeze dryer and shielded by placebo vials (final formulation #2-m without GM-CSF) to mimic large scale conditions. Samples were freeze dried using a conservative prototype lyo-cycle as applied in Example 5.

In-vial temperatures of the samples were monitored by micro-thermocouples. The chamber pressure was monitored by capacitive pressure sensor and Pirani pressure sensor, and recorded via on-line data acquisition to detect the end of sublimation. Process analytical data was collected and evaluated. The lyophilized vials were closed after venting with nitrogen, and sealed with crimp-caps.

The samples were analyzed with the following methods:

- visual appearance, documented by macro photography
- reconstitution behavior (using 0.33mL WFI)
- pH measurement after reconstitution
- residual water content using a generic Karl-Fischer oven method (in duplicate)
- aggregation status by SE-HPLC
- degradation profile by RP-HPLC
- nano-particle analysis by DLS
- additional: nano-particle analysis by MFI.

2. Materials and Apparatus

2.1 Substances and Materials

Table 97 - Substances

Substances	**Lot no.:**	**Manuf. / Suppl.**	**Order no.:**
Control Sample 3	170118/1167/095	-	-
Purified water (≤ 0.2 µmS/cm, < 10 ppb TOC)	-	PJP	-
Kolliphor® P 188, Poloxamer, Ph.Eur.	WPDH566B	BASF	50259527
ortho-Phosphorsäure 85% Ph.Eur.	306246347	Carl Roth GmbH	2608.1
Sodium chloride, EMPROVE® exp Ph.Eur. BP, USP	K45515600	Merck Chemicals	106400
Sucrose, EMPROVE® exp. Ph.Eur.	K427844532005	Merck Chemicals	107653
D(-)-Mannitol, ≥98 %, Ph.Eur., USP	117256176	Carl Roth GmbH	4175
Sodium hydroxide solution, 30 % w/w pure, pharma grade	0000955695	AppliChem	144320.1211
Potassium hydroxide, Ph.Eur.	B03389302	Merck Chemicals	105032
Trifluoroacetic acid, ≥ 99.9 %, for peptide synthesis	245230492	Carl Roth GmbH	P088.1
Acetonitrile, Chromasolv gradient grade	SZBG041AV	Sigma-Aldrich	34851
Materials			

Slide-A-Lyzer dialysis cassettes, 3 mL; MWCO: 3.5 kDa	00601337	Thermo Scientific	87723
Minisart® PES 0.1 µm syringe filter	71112103	Sartorius stedim	16553-K
Jupiter 5 µm C18 (300 Å; 250 × 4.6 mm)	H16-035225	Phenomenex	00G-4053-E0
Superdex™ 200 Increase 10/300 GL	10246668	GE Healthcare	28-990o9-44
2R FIOLAX injection vials, clear	162263031	Nipro Germany GmbH	-
Lyo stopper, 1356 4023/50/GREY B2-TR Westar®RS	1152022693	West	7002-1252

* bulk drug substance from pilot scale used for formulation of the final formulation variants #1-m, #2-m, #1d-m and #2d-m in this study (5 mg/mL GM-CSF, 20 mM K-phosphate, pH 7.2)

2.2 Apparatus

The following apparatus and equipment was used:

Purified water supply

- Manufacturer: Siemens
- Type: Ultra Clean UV UF TM

UV Spectrophotometer

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: Cary 60 UV-Vis
- SN: MY16170003

HPLC Equipment

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: 1100 Series
- Degasser: G1322A
- BinPump : G1312A
- ALS: G1329A
- ALSTherm: G1330A
- ColCom: G1316A
- DAD: G1315A

Freeze dryer (GT1):

- Manufacturer: Hof Sonderanlagenbau (Lohra, Germany)
- 0.5 m^2 shelf area total, 3 shelves
- 10 kg ice condenser capacity
- Adjustable ice condenser temperature
- In-vial temperature recording
- Differential pressure measurement

Micro-Flow Imaging (MFI):

- Manufacturer: Brightwell Technologies Inc.
- Type: DPA 4200
- SN: 000784

Camera Equipment

- Manufacturer: Canon (Tokio, Japan)
- Body: EOS 550D/EOS 600D
- Lens: EFS 18-55mm, DGMacro 105mm 1:2.8

DLS plate reader

- Manufacturer: Wyatt Technology Corporation (Santa Barbara, CA, USA)
- Type: Dynapro plate reader II

Additional Laboratory Equipment

- Analytical balance (Kern)
- Magnetic stirrer (IKA Werke)
- pH meter (Mettler Toledo)
- Volumetric pipets (Gilson)
- Multistep Pipettes: Brand, HandyStep
- Autoclave: Systec, DX65
- Drying oven: Memmert, 500
- Orbital shaker: Wisd Laboratory Instruments, WiseShake SHO-1D
- Laminar flow microbiological class 2 work bench: BDK, UVF 6.18S

3. Methods

3.1 Preparation of liquid bulk drug substance

Sterilization of vials and stoppers:

2R glass vials were washed with purified water and subsequently depyrogenized and sterilized by dry heat at 300 °C for 2 hours.

Stoppers were autoclaved at 134 °C for 10 minutes and subsequently dried at 70 °C for 8 hours.

Preparation of formulation buffers:

Table 98 – Preparation of formulation buffers

Final formulation variant #	**Composition**	**Preparation procedure**
1-m	10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol, pH 7.3	1.15g of 85 % (w/w) ortho-phosphoric acid, 19.85g of sucrose, and 40.08g of mannitol were diluted in 900mL of purified water. Subsequently the pH was adjusted to 7.3 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
2-m	10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol pH 8.1	1.15g of 85% (w/w) ortho-phosphoric acid, 19.85g of sucrose, and 40.08 g of mannitol were diluted in 900mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.

Dialysis:

The dialysis of GM-CSF drug substance (i.e. unformulated GM-CSF) was accomplished in three independent dialysis steps to achieve a quantitative buffer exchange. 2 mL of the GM-CSF drug substance (c = 5.0 mg/mL) were transferred into preconditioned (in dialysis buffer) Slide-A-Lyzer cassettes with a molecular weight cut off of 3.5 kDa and a maximum fill volume of 3mL (Thermo Scientific, Rockford, USA). Filled Slide-A-Lyzer cassettes were incubated in 333mL of the target buffer for 2 hours before a first buffer change (333mL) was performed. After dialysis for two further hours the buffer was exchanged a second time (333mL) to finalize the dialysis overnight. The protein sample was removed from the Slide-A-Lyzer cassettes and processed further. Each dialysis step represents a 1/167 fold buffer exchange leading to a calculated buffer exchange factor of $1:4.7\times10^6$ in total. All dialysis steps were performed at 2-8°C.

Compounding and Filling:

After dialysis the GM-CSF solution was analyzed via UV absorption measurement at 280 nm to determine the GM-CSF concentration. The solution was then diluted to a concentration of 0.3

mg/mL using formulation buffer before it was spiked with the required amount of poloxamer 188 to obtain a concentration of 0.1% (w/v) detergent resulting in final formulation variants #1-m and #2-m. In order to obtain the respective diluted final formulation variants #1d-m and #2d-m, portions of the readily compounded final formulation variants #1-m and #2-m were diluted with the same volume of water (1v+1v). Control variant #2v-m (vehicle/placebo formulation) was formulation buffer #2-m with 0.1% (w/v) poloxamer 188 but without GM-CSF.

The readily compounded lyo solutions were sterile filtered using a 0.1µm PES syringe filter and filled into sterile 2R glass vials by aliquots of 330µL (final formulation variants #1 and #2, and control variant #2v) and 660µL (final formulation variants #1d and #2d), respectively, using a multistep pipette. Subsequently the vials were partially stoppered with lyo stoppers in lyo position and placed in the freeze dryer. All compounding/filling steps were performed under a laminar flow bench to minimize microbial contamination of the product.

3.2 Lyophilization

The filled vials with lyo-stoppers attached to the vials in “lyo-position” (first snap off lyo stopper legs onto the vial, leaving lateral openings for flow of sublimation stream) were positioned on the middle shelf of the freeze dryer. 10 vials were loaded per final formulation variant as well as an additional ~50 placebo vials that were placed around active vials. The process parameters applied for lyophilization are listed in Table 99. A so-called radiation cage was employed to actively cool the walls of the lyo chamber in order to simulate large scale conditions.

Table 99 - Lyophilization process parameters applied for lyophilization feasibility testing of GM-CSF formulations with sucrose and mannitol.

Step		**Shelf temperature**	**Pressure**	**Step time**	**Cumulative time**
#	Description	[°C]	[mbar]	[h:min]	[h:min]
1	Loading	20	atm	00:01	0:01
2	Freezing, ramp	-50	atm	01:10	1:11
3	Freezing	-50	atm	02:00	3:11
4	Annealing, ramp	-20	atm	00:30	3:41
5	Annealing	-20	atm	02:00	5:41
6	Freezing, ramp	-50	atm	00:30	6:11

7	Freezing	-50	atm	10:00*	16:30
8	Vacuum Adjustment	-50	1,00E-01	00:30	17:00
9	Primary Drying, ramp	-10	1,00E-01	01:00	18:00
10	Primary Drying	-10	1,00E-01	44:00	62:00
11	Secondary Drying, ramp	35	1,00E-01	06:00	68:00
12	Secondary Drying	35	1,00E-01	15:00	83:00
13	Venting with N2/stopper closing	35	7,50E+02	00:10	83:10

*Due to a technical issue the second freezing step was about 8 hours longer than planned.

To monitor the product temperature during freeze drying, thermo couples were inserted into product vials. Pressure was controlled during lyophilization by using a capacitive pressure sensor (MKS). Pressure regulation was managed via vacuum and dosing valve (nitrogen injection). After completion of secondary drying vials were closed at a pressure of 800 mbar under nitrogen atmosphere. Vials were unloaded from the freeze dryer and crimp capped (using a hand crimping tool).

3.3 Analytical methods

3.3.1 Absorption measurements

Samples were analyzed using a Cary 60 UV-spectrometer (Agilent Technologies, Santa Clara, CA, USA) by absorption measurement at 280 nm for concentration determination. Samples were measured at a concentration of about 1 mg/mL (dilution with formulation buffer) or undiluted if the concentration was below 1 mg/mL using plastic cuvettes with an optical path length of 1.0 cm. The concentration was calculated according to Lambert-Beer´s law using an extinction coefficient of 0.974 mL/(mg*cm).

3.3.2 SE-HPLC (size exclusion chromatography)

Chromatographic parameters:

Mobile phase: 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0

Flow rate: 0.5 mL/min

Temperature: ambient

UV-detection: 214 nm and 280 nm

Injection volume: 100 µL at 0.3 mg/mL GM-CSF

Sample temperature: 4 ± 2 °C

Buffer and sample preparation:

Table 100 – Buffer and sample preparation

Buffer/solution	Composition/Description	Preparation procedure
Mobile phase	0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0	11.53g of 85 % (w/w) phosphoric acid and 5.84g of sodium chloride were topped with 900mL with purified water in a 1000mL glass beaker. The pH was adjusted to 7.0 with sodium hydroxide before the volume was filled up to 1000mL with purified water.
Reference solution	0.3mg/mL GM-CSF in mobile phase	Control Sample 3 was diluted to 0.3mg/mL with mobile phase.
Sample solution	Reconstituted formulation	Samples were measured undiluted at the target GM-CSF concentration.

3.3.3 RP-HPLC (reversed-phase chromatography)

Chromatographic parameters:

Mobile phase A: 0.1 % TFA in purified water

Mobile phase B: 10 % purified water + 0.1 % TFA in ACN

Flow rate: 1.2 mL/min

Temperature: 50 ± 2 °C

UV-detection: 214 nm

Injection volume: 100 µL at 0.5 mg/mL

Sample temperature: 4 ± 2 °C

Gradient:

Table 101 - Gradient

Time [min]	Pump B conc. [%]
0.10	36
30	56

35	100
45	100
50	36
60	36

Buffer and sample preparation:

Table 102 – Buffer and sample preparation

Buffer/solution	**Composition/Description**	**Preparation procedure**
Mobile Phase A	0.1% TFA in purified water	1mL of trifluoroacetic acid was mixed with 1000 mL of purified water.
Mobile Phase B	10% purified water + 0.1 % TFA in ACN	1mL of trifluoroacetic acid was mixed with 100mL of purified water and 900mL of acetonitrile.
Dilution buffer	0.05M phosphate buffer, pH 7.0	2.63g of potassium dihydrogen phosphate and 4.33g of disodium hydrogen phosphate were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.0 using sodium hydroxide and the volume was filled up to 1000mL with purified water.
Reference solution	0.3mg/mL GM-CSF in dilution buffer	GM-CSF Control Sample 3 was diluted to 0.3mg/mL with dilution buffer.
Sample solution	Reconstituted formulation	Samples were measured undiluted at the target GM-CSF concentration.

3.3.4 Nano-particle analysis by DLS (dynamic light scattering)

The principle of the Wyatt DynaPro Plate reader II is based on dynamic light scattering, as described in Example 3.

Sample preparation:

Each sample constituted a 2R vial with lyophilized GM-CSF formulation with a target GM-CSF amount of 0.10mg per vial. After reconstitution with 0.33mL WFI, samples were filled into the wells of the measurement plate by portions of 30µL and subsequently overlaid with silicone oil to avoid evaporation of liquid. No further treatment was applied before measurement.

3.3.5 Sub-visible particle counting by MFI (micro flow imaging)

Particles ≥ 10 µm were counted using a micro flow imaging tool from Brightwell Technologies. Four reconstituted vials were pooled before the DP solution was sucked through a flow cell by a peristaltic pump. Particles were detected by light obscuration using a laser as source.

3.3.6 Process analytics of the lyophilization

The process data of the lyophilization (time, pressure and temperature readings including in-vial product temperature measurements), were logged by a controlling computer and visualized in a graph.

3.3.7 Visual appearance of the lyophilizate

Lyophilizate-containing vials were examined visually. Photographs were taken for documentation.

3.3.8 Karl-Fischer oven method

The residual moisture of the samples was determined via Karl Fischer titration using a 756 Karl Fischer coulometer equipped with a 774 oven sample processor (Metrohm). For each measurement, one lyophilizate of each sample (~ 15-20mg of a lyophilizate) was transferred to a Karl-Fischer glass vial. The exact weight of the lyophilizate was documented. The vial was sealed with a crimp cap and transferred into the oven of the Karl Fischer coulometer which was heated to 100°C. The septum of the crimp cap was penetrated by an injection needle, and the generated water vapour was directly transferred into the titration chamber of the Karl Fischer coulometer via dry nitrogen flow. Empty glass vials were used as blank correction. Samples were analyzed in duplicate.

3.3.9 Reconstitution

The vials were reconstituted by the addition of 330µL WFI (water for injection) using a volumetric pipette after removing the cap and stopper from the vial. The mixture is gently swirled for two minutes by hand and the vials are then allowed to stand at ambient temperature for 11 minutes. The lyophilized cake should reconstitute to a clear solution. Analysis can commence after the standing time, but the time from reconstitution to the start of analysis is noted.

4. Results and discussion

4.1 Lyophilization process online data

The chamber pressure was monitored by capacitive pressure sensor and Pirani pressure sensor and recorded via on-line data acquisition to detect the end of sublimation.

The lyophilization program was completed as planned. The recorded temperature and pressure profile complied with the predefined process parameters. The product temperatures measured via thermo couples indicate the end of the sublimation phase after about 24 hours total process time for both fill volumes (i.e. 0.33mL and 0.66mL) which corresponds to a primary drying time of about 6 hours.

Unfortunately, the thermo couple in the 0.66mL vial had a technical issue which led to extreme spiking (see spikes at around 24h and 68h). Due to the effect being much stronger at the beginning of the process, the data from this thermo couple is not shown for the first 19.5 hours. However, the important part, the end of sublimation, is well visible at around 24 hours. Interestingly, the diluted variants with higher fill volume had about the same sublimation time as the original variants with half the volume.

4.2 Visual appearance of the lyo cakes

After lyophilization all cakes were examined visually with respect to shrinkage and cake defects. Overall, all obtained lyo cakes were in good acceptable shape besides some very mild shrinkage that cannot be prevented completely. However, the shrinkage observed on these mannitol/sucrose formulations could be reduced compared to variants only containing sucrose (see Example 5) and the overall appearance of the cakes was slightly improved, as planned. Exemplary photographs of the lyophilizates are shown in Figure 54.

4.3 Residual water content

The residual water content in the lyophilizates was determined using a generic Karl-Fischer oven method (100 °C). The results are listed in Table 1033.

Table 103: Residual water content by Karl-Fischer oven method

Final formulation variant	Residual water content [%]
#1-m	0.3
#2-m	0.4
#1d-m	0.6

#2d-m	0.6

All measured residual water values in the lyophilized samples were well below 1%. The small differences between variants are insignificant as the error resulting from small sample volume in combination with very low water content is expected to be relatively large.

4.4 Reconstitution behavior

Reconstitution was performed by addition of 0.33mL WFI and subsequent immediate and gentle shaking. The complete dissolution of the lyo cake of all final formulation variants #1-m, #2-m, #1d-m, #2d-m was achieved after about 10 seconds.

4.5 pH after reconstitution

After reconstitution with 0.33mL WFI the pH value of each final formulation variant was determined. The measured values are listed in Table 104.

Table 104: pH values measured after reconstitution

Final formulation variant	**pH after reconstitution**
#1	7.36
#2	7.91
#1d	7.32
#2d	7.90

pH values were in the expected range. Due to decreasing buffer capacity towards higher pH, the variants with pH 8.1 shifted more strongly during the production process (assumedly mainly during dialysis and dilution with water) than variants with pH 7.3 which is closer to the pK_a of phosphoric acid of 7.21.

4.6 Aggregation by SE-HPLC

The aggregation status of GM-CSF samples after reconstitution with 0.33mL WFI was evaluated by SE-HPLC. For reference, untreated GM-CSF DS (Control Sample 3; see Figure 55) was analyzed in parallel. An overlay chromatogram of the four final formulation variants is shown in Figure 56.

All reconstituted drug product samples showed the same peak pattern of one peak of larger aggregates eluting before the main peak and one peak of smaller fragments eluting after the

main peak. The relative main peak areas detected for each individual sample are listed in Table 105.

Table 105 - Relative main peak areas detected by SE-HPLC (aggregate status)

Final formulation variant	**Rel. main peak area**
Reference (Control Sample 3)	100 %
#1	98.97 %
#2	99.58 %
#1d	99.77 %
#2d	99.76 %

4.7 Degradation by RP-HPLC

The degradation (chemical purity) of the samples after reconstitution with 0.33 mL WFI was evaluated by RP-HPLC. For reference, freshly thawed GM-CSF DS (Control Sample 3; see Figure 57) was analyzed in parallel. A chromatogram overlay of all final formulation variants is shown in Figure 58.

The impurity peak profile in all reconstituted GM-CSF drug product samples #1-m, #2-m, #1d-m, #2d-m as well as in the reference material was the same and as shown in Figure 57 and Figure 58. Peaks eluting before the main peak represent fragments that are more hydrophilic than GM-CSF whereas the peak eluting after the main peak represents fragments that are more hydrophobic than GM-CSF. No significant differences with regards to the peak pattern were observed between the variants and Control Sample 3.

The relative main peak areas detected for each individual sample are listed in Table 106.

Table 106 - Relative main peak areas detected by RP-HPLC (chemical degradation)

Final formulation variant	**Rel. main peak area**
Reference (Control Sample 3)	98.17 %
#1	97.71 %
#2	97.84 %
#1d	97.85 %
#2d	97.68 %

4.8 Nano-particle analysis by DLS

To simplify the complex set of data generated with the DLS plate reader, three ranges of particle sizes were defined (i.e. 0.1-10 nm, 10-100 nm, and 100-1000 nm) and the mass fraction of molecules covered by the specific range was calculated. Table 107 shows the mean radius measured in the specific range as well as the related mass fraction.

Table 107 - Nano-particle size and distribution determined by DLS.

Particle size range	Final formulation variant	Mean particle radius [nm]	Mean mass fraction [%]
0.1 – 10 nm	#1	2.0	100
	#2	1.4	99.3
	#1d	1.4	99.5
	#2d	1.1	99.6
10 – 100 nm	#1	-	-
	#2	15	0.7
	#1d	25	0.5
	#2d	21	0.4
100 – 1000 nm	#1	-	-
	#2	-	-
	#1d	-	-
	#2d	-	-

In all samples the vast majority of particles by mass (i.e. > 99 % in average) was 0.1 – 10 nm large. With a molecular weight of 14.6 kDa GM-CSF is a rather small molecule where the mean particle radius would be expected in the measured range between 0.1 – 10 nm, which seems to correlate well with the high amount of mass fraction detected in this range.

4.9 Sub-visible particle counting by MFI

The analytics performed regarding sub-visible particles do not comply with the method of European Pharmacopoeia 2.9.19 (“Particulate Contamination: Sub-visible Particles”) which requires a sample volume of at least 25 mL. However, the acquired data presents a good first

indication for particle pollution in the samples. The particle counts measured by MFI are listed in Table 108.

Table 108 - Particle count by MFI

Final formulation variant	**Particles/mL 10 – 25 µm**	**Particles/mL > 25 µm**
#1-m	14	0
#2-m	15	0
#1d-m	0	0
#2d-m	7	0

For reference, the US Pharmacopoeia (USP) states the following for sub-visible particles:

"Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL: The preparation complies with the test if the average number of particles present in the units tested does not exceed 25 per mL equal to or greater than 10 µm and does not exceed 3 per mL equal to or greater than 25 µm.

Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL: The preparation complies with the test if the average number of particles present in the units tested does not exceed 6000 per container equal to or greater than 10 µm and does not exceed 600 per container equal to or greater than 25 µm."

5. Conclusions

In this study, the lyophilization feasibility of final formulation variants containing mannitol and sucrose as bulking agent was tested using a conservative lyo cycle (primary drying at -10°C and 100µbar). The main aim was to improve the appearance of the lyo cakes compared to the ones obtained in Example 5, where a test lyophilization cycle with formulations containing only sucrose as bulking agent was performed. For reference and comparability a placebo formulation (#2v-m) was freeze dried in the same run.

Visual appearance:

All obtained lyo cakes were in good acceptable shape, except for some very mild shrinkage that cannot be prevented completely. However, the shrinkage observed on these mannitol/sucrose formulations could be reduced compared to variants only containing sucrose (see Example 6) and the overall appearance of the cakes was slightly improved, as planned.

Residual water content:

All measured residual water values in the lyophilized development samples were well below 1 %.

Reconstitution behavior:

The addition of 0.33mL WFI and subsequent immediate and gentle shaking lead to a complete dissolution of all variants in less than 10 seconds.

pH values after reconstitution:

After reconstitution with 0.33mL WFI, all measured pH values were within a range of ±0.2 pH units of the target value (pH 7.3 or 8.1, respectively).

Aggregation status by SE-HPLC:

All final formulation variants showed an aggregate/fragment content of ≤1 percentage point (lowest measured value: final formulation variant #1-m 98.97 %), the reference showed 100% main peak area.

Degradation by RP-HPLC:

All final formulation variants showed a GM-CSF purity of more than 97.5%. The reference DS (Control Sample 3) had a purity of 98.17%.

Nano-particle analysis by DSL:

In all final formulation variants, the vast majority (>99%) of detected particles (with respect to mass) showed a radius of ~2 nm, which is expected for a small protein like GM-CSF.

Sub-visible particle counting by MFI:

In all samples the particle count for particles in the range of 10 – 25µm was well below 25 particles/mL. In the size range above 25µm no particles were detected.

Example 13

1. Scope and objective

In order to evaluate the final formulation in respect to stability during the use by application with a syringe and possible adsorption/degradation of the drug substance to the inner syringe surface with product contact, an in-use stability study using two different types of syringes was performed.

Table 109: Formulations tested for in-use stability

Final formulation variant #	**Composition of liquid bulk drug substance**	**Composition of lyophilized drug product per vial**	**Comment**
1	0.30 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188,	GM-CSF: 0.10 mg Phosphoric acid: 0.32 mg Sucrose: 29.4 P188: 0.33 mg/mL	fill volume: 330 µL

	pH 7.3	Add KOH to pH 7.3	
2	0.30 mg/mL GM-CSF, 10 mM K-phosphate, 260 mM sucrose, 0.1 % (w/v) poloxamer 188, pH 8.1	GM-CSF: 0.10 mg Phosphoric acid: 0.32 mg Sucrose: 29.4 P188: 0.33 mg/mL Add KOH to pH 8.1	fill volume: 330 µL
1-m	0.30 mg/mL GM-CSF, 10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol 0.1 % (w/v) poloxamer 188, pH 7.3	GM-CSF: 0.10 mg Phosphoric acid: 0.32 mg Sucrose: 6.6 mg Mannitol: 13.4 mg P188: 0.33 mg/mL Add KOH to pH 7.3	fill volume: 330 µL
2-m	0.30 mg/mL GM-CSF, 10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol 0.1 % (w/v) poloxamer 188, pH 8.1	GM-CSF: 0.10 mg Phosphoric acid: 0.32 mg Sucrose: 6.6 mg Mannitol: 13.4 mg P188: 0.33 mg Add KOH to pH 8.1	fill volume: 330 µL

2. Materials and apparatus

2.1 Substances and materials

Table 110 – Substances and materials

Substances	**Lot no.:**	**Manuf. / Suppl.**	**Order no.:**
GM-CSF Control Sample 3	170118/1167/095	-	-
Purified water (≤ 0.2 µmS/cm, < 10 ppb TOC)	-	PJP	-
Kolliphor® P 188, Poloxamer, Ph.Eur.	WPDH566B	BASF	50259527
ortho-Phosphorsäure 85% Ph.Eur.	306246347	Carl Roth GmbH	2608.1
Sodium chloride, EMPROVE® exp Ph.Eur. BP, USP	K45515600	Merck Chemicals	106400

Sucrose, EMPROVE® exp. Ph.Eur.	K4278445320 5	Merck Chemicals	107653
D(-)-Mannit, ≥98 %, Ph.Eur., USP	117256176	Carl Roth GmbH	4175
Sodium hydroxide solution, 30 % w/w pure, pharma grade	0000955695	AppliChem	144320.12 11
Potassium hydroxide, Ph.Eur.	B03389302	Merck Chemicals	105032
Trifluoroacetic acid, ≥ 99.9 %, for peptide synthesis	245230492	Carl Roth GmbH	P088.1
Acetonitrile, Chromasolv gradient grade	SZBG041AV	Sigma-Aldrich	34851
Materials			
Slide-A-Lyzer dialysis cassettes, 3 mL; MWCO: 3.5 kDa	00601337	Thermo Scientific	87723
Minisart® PES 0.1 µm syringe filter	71112103	Sartorius stedim	16553-K
Jupiter 5 µm C18 (300 Å; 250 × 4.6 mm)	H16-035225	Phenomenex	00G-4053-E0
Superdex™ 200 Increase 10/300 GL	10246668	GE Healthcare	28-990o9-44
Insuline syringe 0.5 mL, 0.33 mm (29G) x 12.7 mm cannula	7121989	BD Micro-Fine™	320926
Tuberculine syringe 1.0 mL, Norm-Ject®	18A22C8	Henke Sass Wolf	4010-200V0
Sterican® 0.3 x 12 mm 30G cannula	3H30258811	BBraun	4656300

2.2 Apparatus

The following apparatus and equipment was used:

Purified water supply

- Manufacturer: Siemens
- Type: Ultra Clean UV UF TM

UV Spectrophotometer

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: Cary 60 UV-Vis
- SN: MY16170003

HPLC Equipment

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: 1100 Series
- Degasser G1322A
- BinPump G1312A
- ALS G1329A
- ALSTherm G1330A
- ColCom G1316A
- DAD G1315A

Additional Laboratory Equipment

- Analytical balance (Kern)
- Magnetic stirrer (IKA Werke)
- pH meter (Mettler Toledo)
- Volumetric pipets (Gilson)
- Multistep Pipettes: Brand, HandyStep
- Autoclave: Systec, DX65
- Drying oven: Memmert, 500
- Orbital shaker: Wisd Laboratory Instruments, WiseShake SHO-1D
- Laminar flow microbiological class 2 work bench: BDK, UVF 6.18S

3. Methods

3.2 Preparation of liquid bulk drug substance

Preparation of formulation buffers:

Table 111- Preparation of formulation buffers

Formulation variant #	Composition	Preparation procedure
1	10 mM K-phosphate, 260 mM sucrose, pH 7.3	1.15 g of 85 % (w/w) ortho-phosphoric acid and 89.0 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.3 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.

2	10 mM K-phosphate, 260 mM sucrose, pH 8.1	1.15 g of 85 % (w/w) ortho-phosphoric acid and 89.0 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
1-m	10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol, pH 7.3	1.15 g of 85 % (w/w) ortho-phosphoric acid, 19.85 g of sucrose, and 40.08 g of mannitol were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.3 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
2-m	10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol pH 8.1	1.15 g of 85 % (w/w) ortho-phosphoric acid, 19.85 g of sucrose, and 40.08 g of mannitol were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.

Dialysis:

The dialysis of GM-CSF drug substance was accomplished in three independent dialysis steps to achieve a quantitative buffer exchange. 2 mL of the GM-CSF drug substance (c = 5.0 mg/mL) were transferred into preconditioned (in dialysis buffer) Slide-A-Lyzer cassettes with a molecular weight cut off of 3.5 kDa and a maximum fill volume of 3 mL (Thermo Scientific, Rockford, USA). Filled Slide-A-Lyzer cassettes were incubated in 333 mL of the target buffer for 2 hours before a first buffer change (333 mL) was performed. After dialysis for two further hours the buffer was exchanged a second time (333 mL) to finalize the dialysis overnight. The protein sample was removed from the Slide-A-Lyzer cassettes and processed further. Each dialysis step represents a 1/167 fold buffer exchange leading to a calculated buffer exchange factor of $1:4.7\times10^6$ in total. All dialysis steps were performed at 2-8 °C.

Compounding and filling:

After dialysis the GM-CSF solution was analyzed via UV absorption measurement at 280 nm to determine the GM-CSF concentration. The solution was then diluted to a concentration of 0.3 mg/mL using formulation buffer before it was spiked with the required amount of poloxamer 188 to obtain a concentration of 0.1 % (w/v) detergent.

The readily compounded lyo solutions were sterile filtered using a 0.1 µm PES syringe filter and sucked into the respective syringes (with cannula attached) by aliquots of approx. 330 µL.

3.2 In-use stability testing

Filled syringes were stored at 2-8°C and 30°C, respectively. Samples were analyzed prior to storage and after 5h, 20h, and 27h incubation time at the respective temperature. Samples were analyzed by UV absorption measurement at 280nm for concentration determination and by RP-HPLC for chemical degradation monitoring. Table 112 shows the sampling schedule applied for this study.

Table 112 - Sampling schedule for the in-use stability study of GM-CSF final formulations

Presentation: 0.30 mg/mL, 0.33 mL fill, set-up per variant				
Storage Temperature:	**T0 hours**	**T5 hours**	**T20 hours**	**T27 hours**
2-8°C	Syringe A: 1 syringes Syringe B: 1 syringes	Syringe A: 1 syringes Syringe B: 1 syringes	Syringe A: 1 syringes Syringe B: 1 syringes	Syringe A: 1 syringes Syringe B: 1 syringes
30°C		Syringe A: 1 syringes Syringe B: 1 syringes	Syringe A: 1 syringes Syringe B: 1 syringes	Syringe A: 1 syringes Syringe B: 1 syringes
sum	2	4	4	4
			Total syringes per variant:	14

3.3 Analytical methods

3.3.1 Absorption measurements

Samples were analyzed using a Cary 60 UV-spectrometer (Agilent Technologies, Santa Clara, CA, USA) by absorption measurement at 280nm for concentration determination (320nm reference). Samples were measured undiluted at a concentration of about 0.3mg/mL using plastic cuvettes with an optical path length of 1.0cm. The concentration was calculated according to Lambert-Beer´s law using an extinction coefficient of 0.974mL/(mg*cm).

3.3.2 RP-HPLC (reversed-phase chromatography)

Chromatographic parameters:

Mobile phase A: 0.1 % TFA in purified water

Mobile phase B: 10 % purified water + 0.1 % TFA in ACN

Flow rate: 1.2 mL/min

Temperature: 50 ± 2 °C

UV-detection: 214nm

Injection volume: 100 µL at 0.5 mg/mL

Sample temperature: 4 ± 2 °C

Gradient:

Table 113 - Gradient

Time [min]	Pump B conc. [%]
0.10	36
30	56
35	100
45	100
50	36
60	36

Buffer and sample preparation

Table 114 – Buffer and sample preparation

Buffer/solution	Composition/Description	Preparation procedure
Mobile Phase A	0.1% TFA in purified water	1mL of trifluoroacetic acid was mixed with 1000mL of purified water.
Mobile Phase B	10% purified water + 0.1% TFA in ACN	mL of trifluoroacetic acid was mixed with 100mL of purified water and 900mL of acetonitrile.
Dilution buffer	0.05M phosphate buffer, pH 7.0	2.63g of potassium dihydrogen phosphate and 4.33g of disodium hydrogen phosphate were diluted in 900mL of purified water. Subsequently the pH was adjusted to 7.0 using sodium hydroxide and the volume was filled up to 1000mL with purified water.

4. Results

In this study, the in-use stability of liquid GM-CSF final formulations was evaluated. Therefore, two different types of syringes were filled with the four different final formulation variants of GM-CSF and incubated at 2-8°C and 30°C, respectively, for 5h, 20h, and 27h. Samples were analyzed with respect to GM-CSF concentration by UV-absorption measurement (280nm) and by RP-HPLC in order to monitor chemical degradation.

4.1 GM-CSF concentration by UV-absorption measurement

GM-CSF concentration evaluation was carried out by absorption measurement at 280 nm (320 nm reference) using an extinction coefficient of 0.974 mL/(mg*cm). The results are displayed in Table 115- Table 118.

Table 115 - Norm-Ject tuberculin syringe at 2-8 °C

Time [h]	**#1**	**#2**	**#1-m**	**#2-m**
0	0.30	0.30	0.31	0.30
5	0.30	0.30	0.31	0.30
20	0.30	0.30	0.31	0.31
27	0.29	0.30	0.30	0.30

Table 116 - Norm-Ject tuberculin syringe at 30 °C

Time [h]	**#1**	**#2**	**#1-m**	**#2-m**
0	0.30	0.30	0.31	0.30
5	0.30	0.30	0.31	0.30
20	0.30	0.31	0.32	0.30
27	0.30	0.30	0.31	0.32

Table 117 - Micro-Fine insulin syringe at 2-8 °C

Time [h]	**#1**	**#2**	**#1-m**	**#2-m**
0	0.30	0.30	0.31	0.30
5	0.30	0.30	0.31	0.30
20	0.30	0.30	0.31	0.31
27	0.30	0.30	0.31	0.31

Table 118 - Micro-Fine insulin syringe at 30 °C

Time [h]	#1	#2	#1-m	#2-m
0	0.30	0.30	0.31	0.30
5	0.30	0.30	0.31	0.30
20	0.33	0.31	0.31	0.31
27	0.31	0.32	0.33	0.31

All samples showed a GM-CSF concentration of about 0.3mg/mL. No decrease in GM-CSF concentration could be detected in any sample.

However, six samples showed a somewhat elevated turbidity at the reference wavelength of 320nm. The samples and the respective measured absorbance values at 320nm are listed in Table 119.

Table 119 - Samples with elevated absorbance at the reference wavelength 320 nm

Item #	Sample	Absorbance at 320 nm
	Buffer blank	0.095 – 0.105
1	#1 insulin, T20 at 2-8 °C	0.120
		0.116
2	#2-m insulin, T20 at 2-8 °C	0.128
		0.097
3	#1 insulin, T20 at 30 °C	0.285 (strong turbidity visible)
		0.279 (strong turbidity visible)
4	#1 insulin, T27 at 2-8 °C	0.113
		0.111
5	#1 insulin, T27 at 30 °C	0.111
		0.114
6	#2 insulin, T27 at 30 °C	0.128
		0.130
7	#1-m insulin, T27 at 30 °C	0.122
		0.117

Item #3 showed strong turbidity, well visible with the bare eye, and it seems to be an outlier. Only samples incubated in insulin syringes showed any elevated turbidity.

4.2 Chemical degradation by RP-HPLC

Chemical degradation of GM-CSF was monitored by RP-HPLC. The results are displayed in Figures 59 - 62. In all samples stored at 30°C, a decrease in purity by 0.4-0.9 percentage points compared to T=0 could be detected. T=0 samples showed the same purity as freshly thawed formulations of about 98.2%. Samples stored at 2-8°C did not show any significant decrease in purity.

5. Conclusions

The in-use stability of four different final formulation variants of GM-CSF liquid drug product was evaluated in two different syringe types, at 2-8°C and 30°C, respectively, over the course of 27 hours. Syringes were filled through the attached cannulas by aliquots of about 330µL liquid GM-CSF final formulation variants and subsequently stored at the respective incubation temperature. Samples were analyzed by UV-absorption measurement to determine the GM-CSF concentration as well as by RP-HPLC in order to monitor chemical degradation.

Overall, no significant reduction in GM-CSF concentration could be detected in any sample. However, some samples stored in insulin syringes showed a somewhat elevated turbidity at the reference wavelength of 320nm at both temperatures after 20h and 27h of incubation time, respectively. This might be due to an incompatibility between the plunger and/or the silicone oil of the syringe and GM-CSF.

Chemical degradation could only be detected for samples stored at 30°C, after 20h and 27h, respectively. Relative main peak areas decreased by 0.4-0.9 percentage points (compared to T0) across all variants without significant difference between the variants.

Example 14

1. Scope and Objectives

This study aimed to evaluate the stability of GM-CSF in four potential formulations (#1m, #1m-d, #2m and #2m-d) for lyophilization after some adjustments subsequent to Example 2.

Table 120 – Final formulation variants tested by forced degradation

Final Formulation Variant #	**Formulation**	**Comment**
#2	10 mM K-phosphate, 260 mM sucrose, 0.1 % poloxamer 188, pH 8.1	Only for T=0 analytics to serve as reference.
#2-d	5 mM K-phosphate, 130 mM sucrose, 0.1 % poloxamer 188, pH 8.1	Only for T=0 analytics to serve as reference.
#1m	10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol, 0.1 % poloxamer 188, pH 7.3	Successor of variant #1, containing mannitol as bulking agent in addition to sucrose for optimization of lyo cake appearance.
#1m-d	5 mM K-phosphate, 29 mM sucrose, 110 mM mannitol, 0.1 % poloxamer 188, pH 7.3	#1m diluted with water to achieve higher fill volume for lyophilization.
#2m	10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol 0.1 % poloxamer 188, pH 8.1	Successor of variant #2, containing mannitol as bulking agent in addition to sucrose for optimization of lyo cake appearance.
#2m-d	5 mM K-phosphate, 29 mM sucrose, 110 mM mannitol, 0.1 % poloxamer 188, pH 8.1	#2m diluted with water to achieve higher fill volume for lyophilization.

The final formulation variants were prepared with a GM-CSF target concentration of 0.3 mg/mL and spiked with poloxamer 188. The resulting final formulation variants were enclosed in lyo vials with coated serum stoppers. In addition to the described four final formulation variants, final formulation variants #2 and #2-d (containing sucrose as bulking agent only) was prepared for T0 analytics and comparison.

2. Materials and Apparatus

2.1 Materials and Substances

Table 121 – Materials and substances

Substances	**Lot no.:**	**Manuf. / Suppl.**	**Order no.:**
GM-CSF Control Sample 3	170118/1167/095	-	-
Purified water (≤ 0.2 µmS/cm, < 10 ppb TOC)	-	PJP	-
Kolliphor® P 188, Poloxamer, Ph.Eur.	WPDH566B	BASF	50259527
ortho-phosphoric acid 85 % Ph.Eur.	306246347	Carl Roth GmbH	2608.1
Sodium chloride, EMPROVE® exp Ph.Eur. BP, USP	K45515600	Merck Chemicals	106400
Sucrose, EMPROVE® exp. Ph.Eur.	K42784453205	Merck Chemicals	107653
D(-)-mannitol, ≥98 %, Ph.Eur., USP	117256176	Carl Roth GmbH	4175
Sodium hydroxide solution, 30 % w/w pure, pharma grade	0000955695	AppliChem	144320.1211
Potassium hydroxide, Ph.Eur.	B03389302	Merck Chemicals	105032
Trifluoroacetic acid, ≥ 99.9 %, for peptide synthesis	245230492	Carl Roth GmbH	P088.1
Materials			
Slida-A-Lyzer dialysis cassettes, 3 mL; MWCO: 3.5 kDa	00601337	Thermo Scientific	87723
Minisart® PES 0.1 µm syringe filter	71112103	Sartorius stedim	16553-K
Jupiter 5 µm C18 (300 Å; 250 × 4.6 mm)	H16-035225	Phenomenex	00G-4053-E0

Superdex™ 200 Increase 10/300 GL	10246668	GE Healthcare	28-990o9-44
2R FIOLAX injection vials, clear	162263031	Nipro Germany GmbH	-
Serum stoppers, 1358 4023/50/G B2-40 Westar®RS SQS	1162061988	West	7002-3049
Crimp caps, aluminum, silver	1306241600	Dätwyler Pharma Packaging	-

2.2 Apparatus

The following apparatus and equipment was used:

Purified water supply:

- Manufacturer: Siemens
- Type: Ultra Clean UV UF TM

UV Spectrophotometer

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: Cary 60 UV-Vis
- SN: MY16170003

HPLC Equipment

- Manufacturer: Agilent Technologies (Santa Clara, CA, USA)
- Type: 1100 Series
- Degasser G1322A
- BinPump G1312A
- ALS G1329A
- ALSTherm G1330A
- ColCom G1316A
- DAD G1315A

Suntest

- Manufacturer: Atlas Material Testing Technology (Linsengericht-Altenhasslau, Germany)
- Type: Suntest CPS+

Pilot freeze dryer (GT1)

- Manufacturer: Hof Sonderanlagenbau (Lohra, Germany)
- 0.5 m^2 shelf area
- In-vial temperature recording
- Differential pressure measurement

Additional Laboratory Equipment

- Analytical balance (Kern)
- Magnetic stirrer (IKA Werke)
- pH meter (Mettler Toledo)
- Volumetric pipets (Gilson)
- Multistep Pipettes: Brand, HandyStep
- Autoclave: Systec, DX65
- Drying oven: Memmert, 500
- Orbital shaker: Wisd Laboratory Instruments, WiseShake SHO-1D
- Climate chamber: Binder
- Laminar flow microbiological class 2 work bench: BDK, UVF 6.18S

3. Methods

3.1 Forced Degradation Study

3.1.1 Preparation of final formulation variants

Sterilization of vials and stoppers:

2R glass vials were washed with purified water and subsequently depyrogenized and sterilized by dry heat at 300 °C for 2 hours. Stoppers were autoclaved at 134 °C for 10 minutes and subsequently dried at 70 °C for 8 hours.

Table 122 - Preparation of formulation buffers and detergent/stabilizer stock solutions

Final Formulation Variant	**Composition**	**Preparation procedure**
#2	10 mM K-phosphate, 260 mM sucrose, pH 8.1	1.15 g of 85 % (w/w) ortho-phosphoric acid and 89.0 g of sucrose were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
#1m	10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol, pH 7.3	1.15 g of 85 % (w/w) ortho-phosphoric acid, 19.85 g of sucrose, and 40.08 g of mannitol were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.3 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.

#2m	10 mM K-phosphate, 58 mM sucrose, 220 mM mannitol, pH 8.1	1.15 g of 85 % (w/w) ortho-phosphoric acid, 19.85 g of sucrose, and 40.08 g of mannitol were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 8.1 using potassium hydroxide and the volume was filled up to 1000 mL with purified water.
Poloxamer 188 stock solution	10 % (w/v) poloxamer 188	1 g of poloxamer 188 was filled up to 10 mL total volume with purified water and stirred until complete dissolution.

Dialysis:

The dialysis of GM-CSF drug substance was accomplished in three independent dialysis steps to achieve a quantitative buffer exchange. 3 mL of the GM-CSF drug substance (c = 5.0 mg/mL) were transferred into preconditioned (in dialysis buffer) Slide-A-Lyzer cassettes with a molecular weight cut off of 3.5 kDa and a maximum fill volume of 3 mL (Thermo Scientific, Rockford, USA). Filled Slide-A-Lyzer cassettes were incubated in 330 mL of the target buffer for 2 hours before a first buffer change (330 mL) was performed. After dialysis for two further hours the buffer was exchanged a second time (330 mL) to finalize the dialysis overnight. The protein sample was removed from the Slide-A-Lyzer cassettes and processed further. Each dialysis step represents a 1/110 fold buffer exchange leading to a calculated buffer exchange factor of $1:1.3\times10^6$ in total. All dialysis steps were performed at 2-8 °C.

Compounding and Filling:

After dialysis the GM-CSF solution was analyzed via UV absorption measurement at 280 nm in order to determine the GM-CSF concentration. The solution was then diluted to the target concentration for the forced degradation study of 0.3 mg/mL using formulation buffer. Final formulation variants #1m-d and #2m-d were further diluted to a GM-CSF concentration of 0.15 mg/mL using water. Subsequently the solutions were spiked with poloxamer 188 stock solution to obtain a concentration of 0.1 % (w/w) detergent.

The readily compounded final formulation variant solutions were sterile filtered using a 0.1 µm PES syringe filter and filled into sterile 2R glass vials by aliquots of 700 µL using a multistep pipette. Subsequently the vials were partially stoppered with serum stoppers and crimped with 13 mm crimp caps. All compounding/filling steps were performed under a laminar flow bench to avoid microbial contamination of the product.

3.1.2 Stress conditions and sampling points

For sampling and analysis the crimp caps and stoppers were removed from the respective vials before the appropriate volumes of samples were taken for the different analytical methods: 150 µL for SE-HPLC, 150 µL for RP-HPLC, 150 µL for UV absorption measurements.

T=0:

Sampling right after compounding and filling without any further stress.

Freeze/Thaw (F/T):

Vials were placed in a freeze dryer in upright position and the program listed in Table 123 was applied.

Table 123 - Lyo parameters for application of the freeze/thaw stress conditions.

Step	**Start temp [°C]**	**End temp [°C]**	**Scanning rate [°C/min]**	**Duration [min]**
Loading	+25	+25	-	1
Freezing, ramp	+25	-50	1	75
Freezing	-50	-50	-	15
Thawing, ramp	-50	+25	1	75
Thawing	+25	+25	-	15
Freezing, ramp	+25	-50	1	75
Freezing	-50	-50	-	15
Thawing, ramp	-50	+25	1	75
Thawing	+25	+25	-	15
Freezing, ramp	+25	-50	1	75
Freezing	-50	-50	-	15
Thawing, ramp	-50	+25	1	75
Thawing	+25	+25	-	15

Light stress (Sun):

Vials were treated in the sun tester at 750 W/m^2 and 35 °C for 7.5 hours in upside-down position.

Agitation (Shake):

Vials were treated on a shaker at 200 rpm and 25 °C for 5 days in upside-down position.

Thermal stress (40 °C):

Vials were stored at 40 °C for 5 days in upside-down position.

3.2 Analytical method

3.2.1 Absorption measurements

Samples were analyzed using a Cary 60 UV-spectrometer (Agilent Technologies, Santa Clara, CA, USA) by absorption measurement at 280 nm for concentration determination as well as at 350 nm and 510 nm for the evaluation of potential turbidity caused by the stress treatment. Samples were measured at a concentration of about 1 mg/mL (dilution with formulation buffer) or undiluted if the concentration was below 1 mg/mL using plastic cuvettes with an optical path length of 1.0 cm. The concentration was calculated according to Lambert-Beer´s law using an extinction coefficient of 0.974 mL/(mg*cm).

3.2.2 SE-HPLC (size exclusion chromatography) with Superdex S200

Chromatographic parameters:

Mobile phase: 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0

Flow rate: 0.5 mL/min

Temperature: ambient

UV-detection: 214 nm and 280 nm

Injection volume: 100 µL at 0.3 mg/mL GM-CSF

Sample temperature: 4 ± 2 °C

Table 124 – Buffer and sample preparation

Buffer/solution	Composition/Description	Preparation procedure
Mobile phase	0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.0	11.53 g of 85 % (w/w) phosphoric acid and 5.84 g of sodium chloride were topped with 900 mL with purified water in a 1000 mL glass beaker. The pH was adjusted to 7.0 with sodium hydroxide before the volume was filled up to 1000 mL with purified water.
Sample solution	Stressed samples	Stressed samples were measured undiluted at the target GM-CSF concentration of about 0.3 mg/mL.

3.2.3 RP-HPLC (reversed-phase chromatography)

Chromatographic parameters:

Mobile phase A: 0.1 % TFA in purified water

Mobile phase B: 10 % purified water + 0.1 % TFA in ACN

Flow rate: 1.2 mL/min

Temperature: 50 ± 2 °C

UV-detection: 214 nm

Injection volume: 100 µL at 0.5 mg/mL

Sample temperature: 4 ± 2 °C

Table 125 – Gradient

Time [min]	Pump B conc. [%]
0.10	36
30	56
35	100
45	100
50	36
60	36

Table 126 – Buffer and sample preparation

Buffer/solution	Composition/Description	Preparation procedure
Mobile Phase A	0.1 % TFA in purified water	1 mL of trifluoroacetic acid was mixed with 1000 mL of purified water.
Mobile Phase B	10 % purified water + 0.1 % TFA in ACN	1 mL of trifluoroacetic acid was mixed with 100 mL of purified water and 900 mL of acetonitrile.
Dilution buffer	0.05 M phosphate buffer, pH 7.0	2.63 g of potassium dihydrogen phosphate and 4.33 g of disodium hydrogen phosphate were diluted in 900 mL of purified water. Subsequently the pH was adjusted to 7.0 using sodium hydroxide and the volume was filled up to 1000 mL with purified water.
Sample solution	Stressed samples	Stressed samples were measured undiluted at the target GM-CSF concentration of about 0.3 mg/mL.

4. Results and discussion

Four final formulation variants (#1m, #1m-d, '2m and #2m-d, shown in Table 120) were evaluated with respect to chemical stability and aggregation status after applying four different stress conditions within the course of a forced degradation study. Final formulation variants were exposed to the stress conditions as summarized in the set-up in Table 127 below.

Table 127 - Forced degradation sampling points for all variants

Presentation per formulation variant, fill 0.7 mL				
	T0	**T7.5 hours**	**After freeze-thaw**	**T5 days**
40 °C	2 vials (+2 spare vials)			2 vials (+2 spare vials)
Agitation				2 vials (+2 spare vials)
Exposure to light		2 vials (+2 spare vials)		
Freeze-thaw			2 vials (+2 spare vials)	
sum	4 vials	4 vials	4 vials	8 vials
			Total	**22 vials per variant**

4.1 Concentration and turbidity by absorption mesaurements

Absorption was measured at 280 nm in order to evaluate the GM-CSF concentration after stress application as well as at 350 nm and 510 nm to detect any increase in turbidity. The determined concentrations are listed in Table 128.

Table 128 - GM-CSF concentrations (mg/mL) determined by UV absorption measurement at 280 nm

	#2	**#2-d**	**#1m**	**#1m-d**	**#2m**	**#2m-d**
T=0	0.31	0.15	0.33	0.16	0.32	0.16
F/T	n.d.	n.d.	0.33	0.16	0.33	0.16
Sun	n.d.	n.d.	0.33	0.16	0.32	0.16
Shake	n.d.	n.d.	0.34	0.18	0.34	0.16
40 °C	n.d.	n.d.	0.33	0.17	0.33	0.16

(n.d.: not determined)

For a better overview and comparability, the results were normalized taking the concentrations determined at T=0 as reference. The calculated relative concentrations are shown in Figure 63.

By UV absorption measurement only, a minor decrease in GM-CSF concentration of up to 2 % could be detected, and only after exposure to light. All other stress conditions did not cause any detectable decrease in concentration. The small concentration decrease observed in the light stressed samples, however, is not significant enough for a discriminating comparison of the final formulation variants. In all samples no significant absorbance (all ≤ 0.01 AU) at 350 nm and 510 nm, respectively, was measured.

4.2 Aggregate status by SE-HPLC

The aggregation status of the samples after stress application was determined by SE-HPLC. The relative main peak areas detected in all samples are summarized inTable 129.

Table 129 - Relative main peak areas by SE-HPLC

	#2	**#2-d**	**#1m**	**#1m-d**	**#2m**	**#2m-d**
T=0	99.84	99.92	99.92	99.92	99.88	99.91
F/T	n.d.	n.d.	99.89	99.88	99.86	99.86
Sun	n.d.	n.d.	99.75	99.49	98.94	98.93
Shake	n.d.	n.d.	99.83	99.90	99.88	99.80
40 °C	n.d.	n.d.	99.81	99.80	99.82	99.79

For a better overview and comparison the above listed values are presented graphically in Figure 64. In light stressed samples, only, an increase in aggregate content by up to 1.1 percentage points could be detected. All other stress conditions did not result in aggregation. Final formulation variants #2m and #2m-d showed slightly more aggregate content than variants #1m and #1m-d, by around 0.5 percentage points. Final formulation variant #1m showed the highest relative main peak area after light stress with 99.8 %.

4.3 Chemical degradation by RP-HPLC

The chemical degradation of the samples after stress application was determined by RP-HPLC. The relative main peak areas detected in all samples are summarized in Table 130.

Table 130 - Relative main peak areas by RP-HPLC

	#2	#2-d	#1m	#1m-d	#2m	#2m-d
T=0	97.75	97.89	98.05	98.03	98.08	97.77
F/T	n.d.	n.d.	98.07	97.86	98.08	97.65
Sun	n.d.	n.d.	85.90	87.81	83.10	82.54
Shake	n.d.	n.d.	96.12	96.27	96.07	96.04
40 °C	n.d.	n.d.	94.04	93.74	93.25	93.48

(n.d.: not determined)

For a better overview and comparison the above listed values are presented graphically in Figure 65.

The RP-HPLC method for evaluation of the chemical degradation yielded the most discriminating results, whereby light stressed samples showed the lowest purities overall. However, freeze/thaw stress can be considered the main concern for a lyophilized product and did not result in any detectable degradation of GM-CSF in any variant. Agitation resulted in a reduction of purity by around 2 percentage points, storage at 40 °C led to a reduction of purity by roughly 4 percentage points. The differences between the final formulation variants were rather small. Final formulation variants #1m and #1m-d showed slightly higher purity than final formulation variants #2m and #2m-d only after light stress. It should be noted, however, that differences of up to 6 percentage points were detected between duplicate vials reducing the significance of differences observed between final formulation variants.

5. Conclusions

In this study, the chemical and colloidal stability of GM-CSF in four different liquid formulations (final formulations #1m, #1m-d, #2m and #2m-d) for lyophilization was evaluated using a forced degradation plan covering four stress conditions listed in Table 127. Samples were analyzed with respect to aggregate status by SE-HPLC, with respect to chemical degradation by RP-HPLC, as well as by light absorption measurement at 280 nm, 350 nm, and 510 nm to monitor GM-CSF concentration and potential turbidity. The results are sorted by method and summarized below.

Absorption measurements at 280 nm, 350 nm, and 510 nm:

- Only light stress led to a decrease in GM-CSF concentration, by up to 2 percentage points (variant #2m), whereby differences between final formulation variants were too insignificant for a discrimination between the final formulation variants.

- No significant turbidity could be detected in any sample (all measured values ≤ 0.01 AU) by absorption measurement at 350 nm and 510 nm.

Aggregate status by SE-HPLC:

- Again, only in the light stressed samples was there an increase in aggregate content, by up to 1.1 percentage points (final formulation variants #2m and #2m-d). Final formulation variant #1m showed the lowest aggregate content with 99.8 % main peak area. However, considering the magnitude of the differences between the final formulation variants no proper discrimination between the final formulation variants can be made.

Chemical degradation by RP-HPLC:

- The biggest impact of the stress application on GM-CSF stability could be detected by RP-HPLC.
- Light stress was, again, identified as the harshest stress condition with reduction of main peak area by up to 15 percentage points, with final formulation variants #2m and #2m-d showing slightly lower purity than final formulation variants #1m and #1m-d. It has to be noted, however, that differences of up to 6 percentage points were detected between duplicate vials, thereby reducing the significance of the apparent differences between final formulation variants greatly.
- Incubation at 40 °C for 5 days led to a decrease of up to 4 percentage points with no significant differences between final formulation variants.
- Agitation at 200 rpm and 25 °C for 5 days led to a decrease in purity by up to 2 percentage points, with no significant differences between final formulation variants.
- Freeze/thaw stress, being arguably the most relevant stress condition for a freeze dried product, did not result in any detectable decrease in GM-CSF purity in any sample.

Final Conclusion:

All tested final formulation variants seem equally feasible for a lyophilized GM-CSF drug product.

CLAIMS:

1. A formulation comprising:

a GM-CSF protein having at least 60% sequence identity to the polypeptide sequence of SEQ ID NO: 2; and

a stabiliser comprising a poloxamer, wherein the poloxamer is present in an amount of 0.5-3.0wt%, excluding water;

wherein the formulation does not comprise more than 0.2wt% of human serum albumin or recombinant human albumin.

2. A formulation according to claim 1, wherein the GM-CSF protein has at least 70%, 80%, 90%, 95% or 99% sequence identity to the polypeptide sequence of SEQ ID NO: 2.

3. A formulation according to claim 1 or 2, wherein the stabiliser further comprises a disaccharide or arginine.

4. A formulation according to claim 3, wherein the disaccharide or arginine is present in an amount of 10.0-99.0wt%, preferably 30.0-99.0wt%, most preferably 95.0-98.0wt%, excluding water.

5. A formulation according to claim 3 or 4, wherein the disaccharide is sucrose and/or mannitol.

6. A formulation according to any one of claims 1-5, wherein the polymer is present in an amount of 1.0-2.5wt%, excluding water.

7. A formulation according to any one of claims 1-6, wherein the poloxamer is P188.

8. A formulation according to any one of claims 1-7, wherein the formulation further comprises a buffer.

9. A formulation according to claim 8, wherein the buffer is present in an amount of 0.5-5.0wt%, preferably 1.0-2.5wt%, excluding water.

10. A formulation according to claim 8 or 9, wherein the buffer is potassium phosphate.

11. A formulation according to any one of claims 1-10, wherein the formulation does not comprise more than 0.02wt% of human serum albumin or recombinant human albumin.

12. A formulation according to any one of claims 1-11, wherein the GM-CSF protein is present in an amount of 0.001-10.0wt%, preferably 0.01-10.0wt%, most preferably 0.1-10.0wt%, excluding water.

13. A formulation according to any one of claims 1-12, wherein the GM-CSF protein has at least 60%, 70%, 80%, 90%, 95%, 99% or 100% sequence identity to SEQ ID NO: 3.

14. A formulation according to any one of claims 1-13, wherein the formulation is liquid.

15. A formulation according to claim 14, wherein the formulation has a pH in the range of 6.5 to 9.0.

16. A formulation according to claim 14 or 15, wherein the formulation has a pH in the range of 7.3 to 8.1, preferably wherein the formulation has a pH of 7.3 or 8.1, and more preferably a pH of 8.1.

17. A formulation according to any one of claims 1-13, wherein the formulation is a frozen liquid.

18. A formulation according to any one of claims 1-17, wherein the formulation comprises water in an amount of 10.0-99.0wt%, preferably 50.0-99.0wt%, and most preferably 90.0-99.9wt%.

19. A formulation according to any one of claims 1-13, wherein the formulation is freeze dried and contains less than 5% residual water.

20. A formulation as defined in any one of claims 1-19, for use as a medicament.

21. A formulation for use according to claim 20, wherein the formulation is for use simultaneously or sequentially with a peptide or protein vaccine.

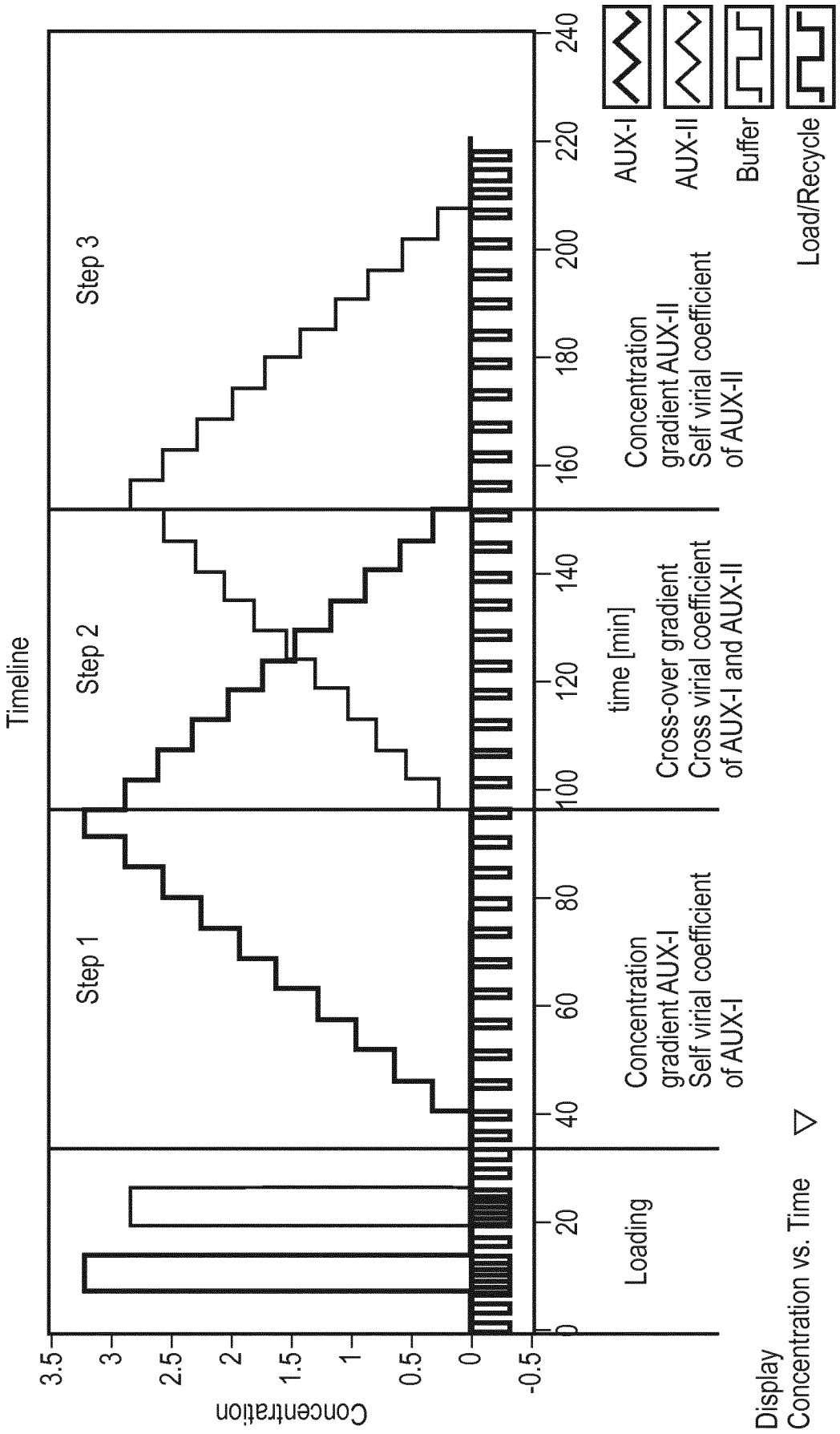
Timeline
Step 1
Step 2
Step 3
Concentration
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
0
20
40
60
80
100
120
140
160
180
200
220
240
time [min]
Loading
Concentration gradient AUX-I
Self virial coefficient of AUX-I
Cross-over gradient
Cross virial coefficient of AUX-I and AUX-II
Concentration gradient AUX-II
Self virial coefficient of AUX-II
AUX-I
AUX-II
Buffer
Load/Recycle
Display
Concentration vs. Time

FIG. 1

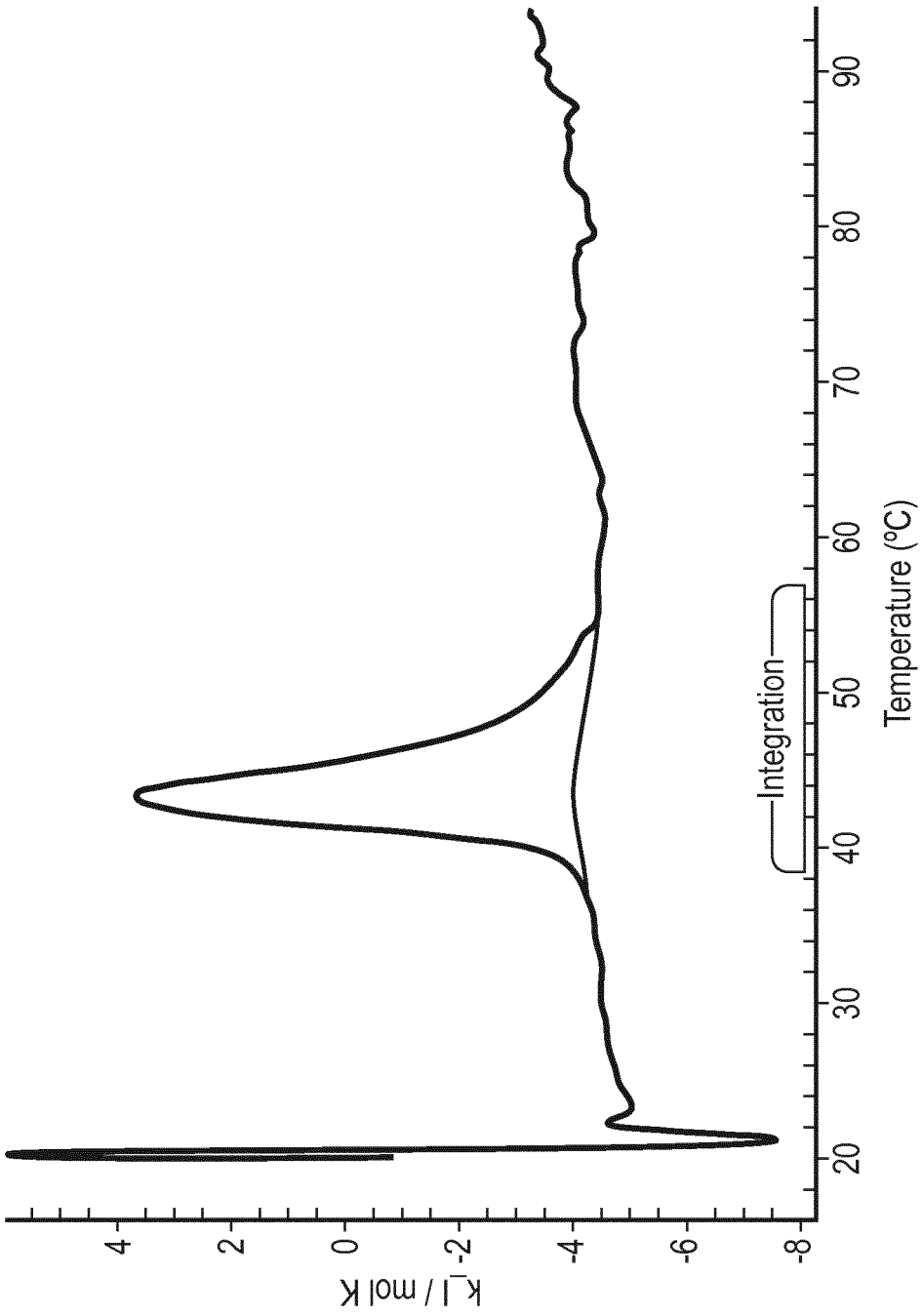
kJ / mol K
4
2
0
-2
-4
-6
-8
20
30
40
50
60
70
80
90
Temperature (°C)
Integration

FIG. 2

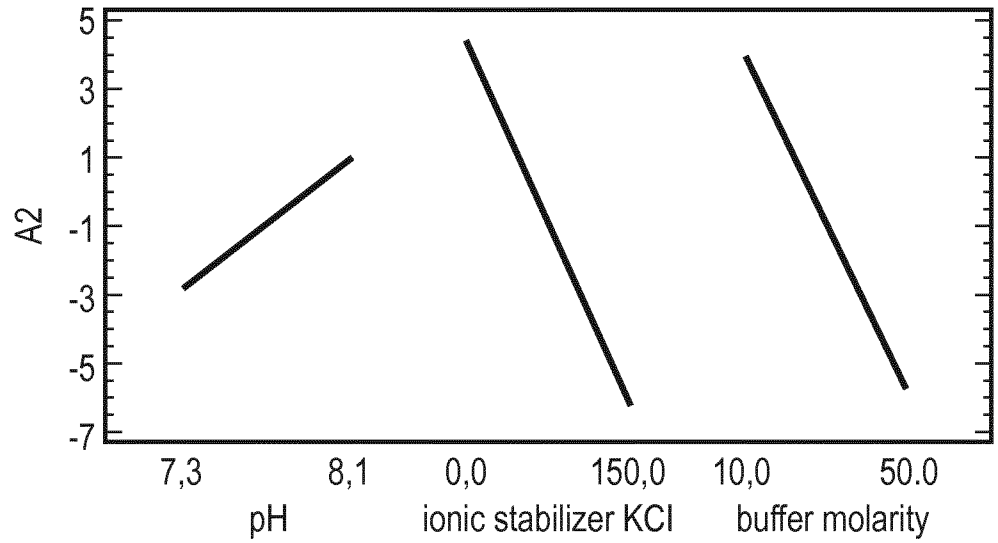
A2
5
3
1
-1
-3
-5
-7
7,3
8,1
pH
0,0
150,0
ionic stabilizer KCl
10,0
50.0
buffer molarity

FIG. 3

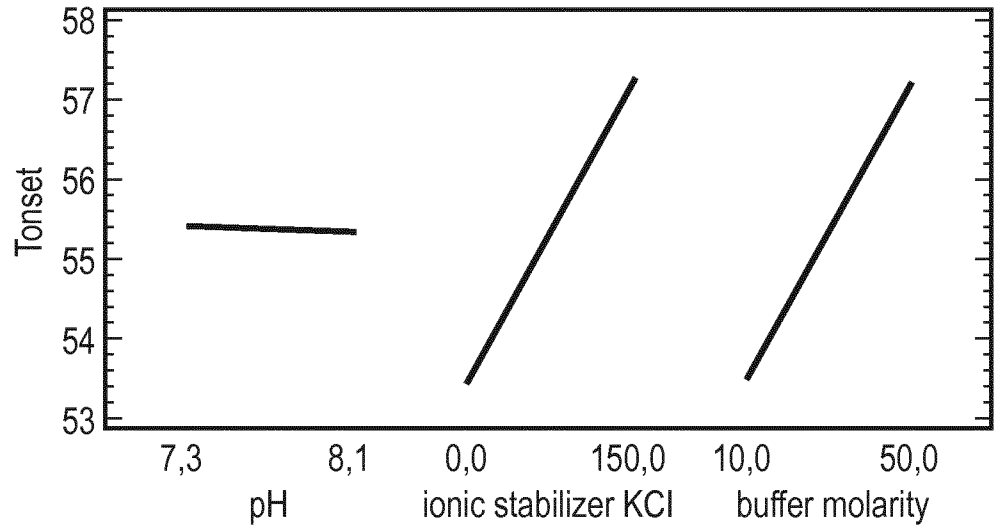
Tonset
58
57
56
55
54
53
7,3
8,1
pH
0,0
150,0
ionic stabilizer KCl
10,0
50,0
buffer molarity

FIG. 4

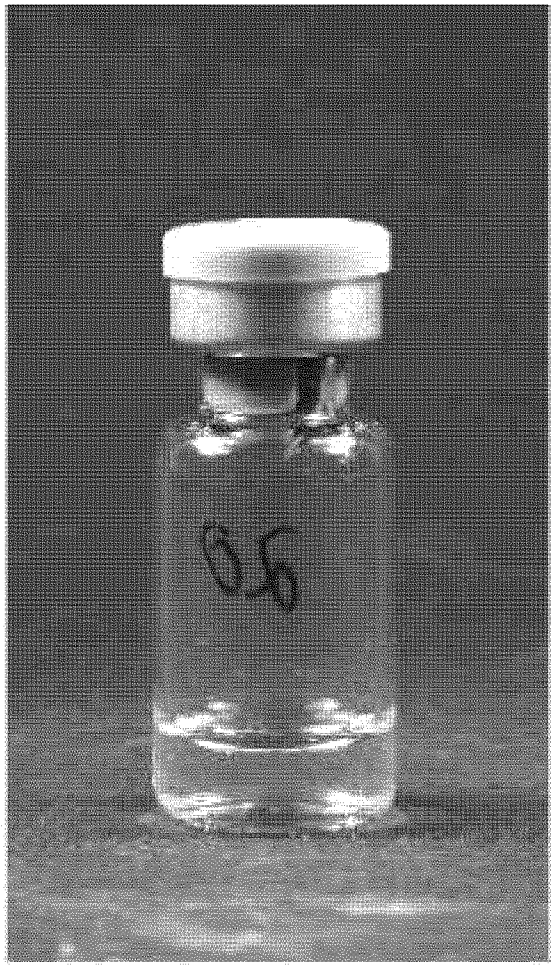

FIG. 5

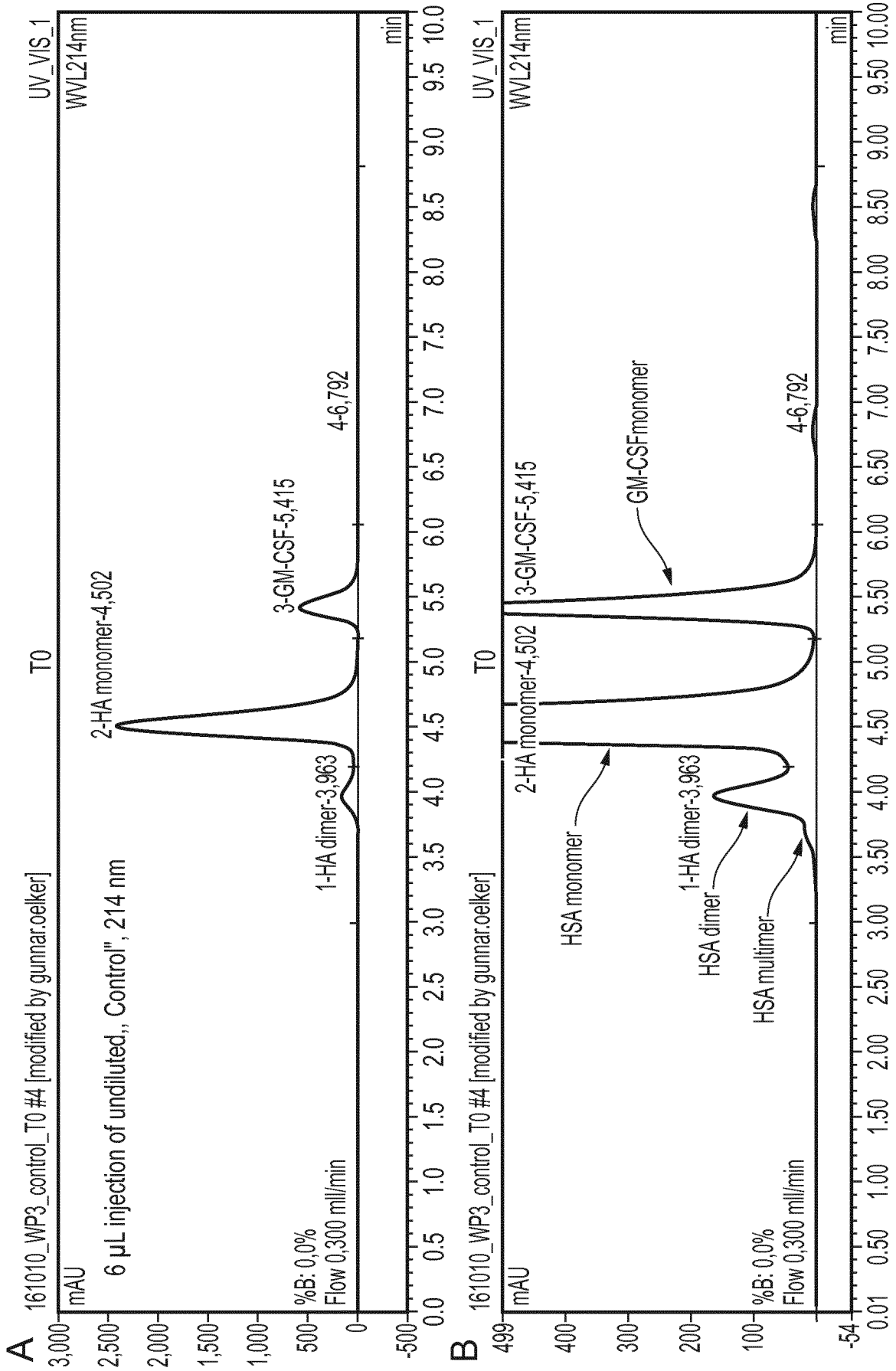
A
161010_WP3_control_T0 #4 [modified by gunnar.oelker]
T0
UV_VIS_1
WVL214nm
mAU
6 µL injection of undiluted,, Control", 214 nm
2-HA monomer-4,502
3-GM-CSF-5,415
4-6,792
1-HA dimer-3,963
%B: 0,0%
Flow 0,300 ml/min
min
B
161010_WP3_control_T0 #4 [modified by gunnar.oelker]
T0
UV_VIS_1
WVL214nm
mAU
2-HA monomer-4,502
3-GM-CSF-5,415
GM-CSFmonomer
4-6,792
HSA monomer
1-HA dimer-3,963
HSA dimer
HSA multimer
%B: 0,0%
Flow 0,300 ml/min
min

FIG. 6

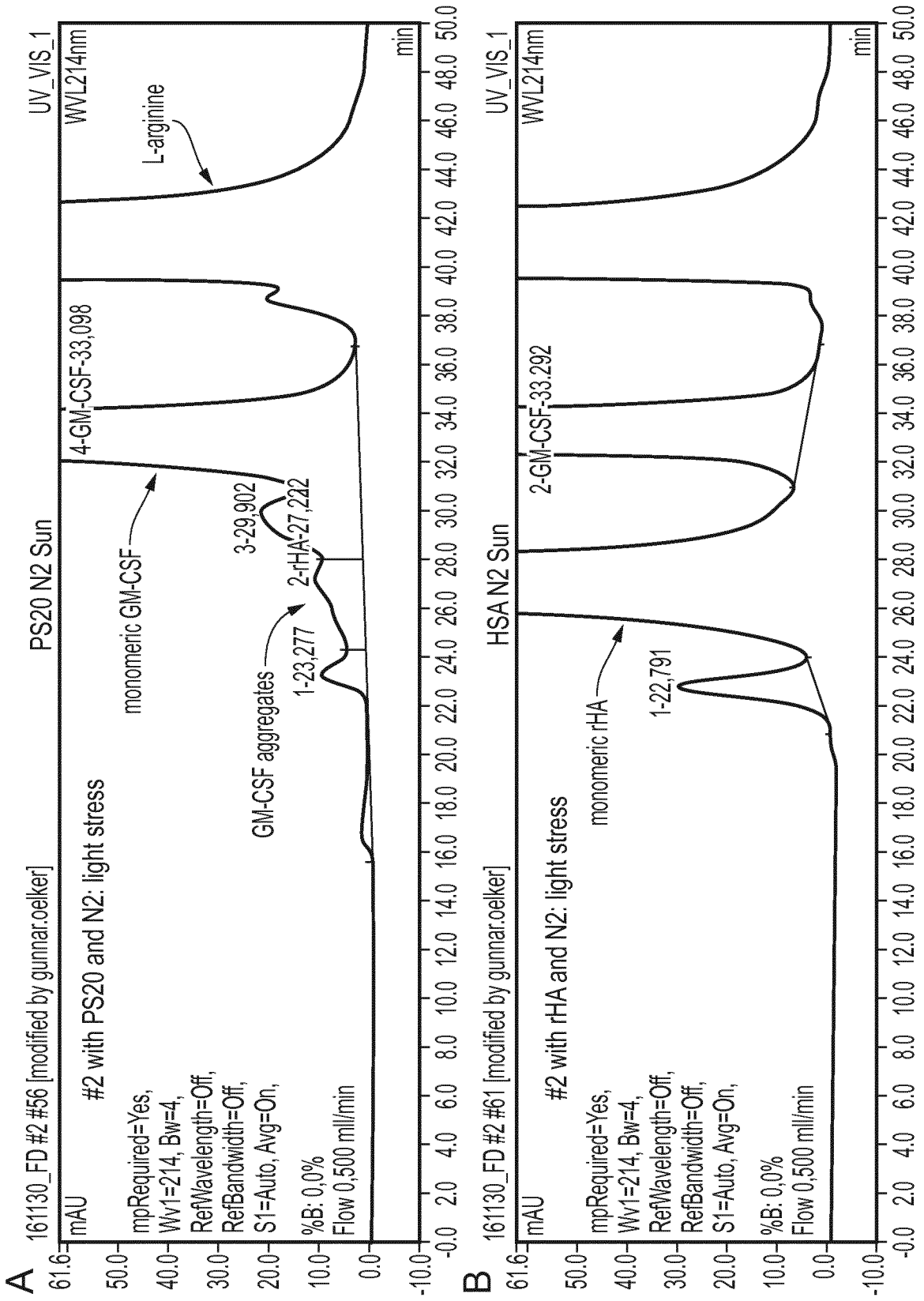
A
161130_FD #2 #56 [modified by gunnar.oelker]
PS20 N2 Sun
UV_VIS_1
WVL214nm
mAU
#2 with PS20 and N2: light stress
mpRequired=Yes,
Wv1=214, Bw=4,
RefWavelength=Off,
RefBandwidth=Off,
S1=Auto, Avg=On,
%B: 0,0%
Flow 0,500 ml/min
monomeric GM-CSF
4-GM-CSF-33,098
L-arginine
3-29,902
2-rHA-27,222
GM-CSF aggregates
1-23,277
min
B
161130_FD #2 #61 [modified by gunnar.oelker]
HSA N2 Sun
UV_VIS_1
WVL214nm
mAU
#2 with rHA and N2: light stress
mpRequired=Yes,
Wv1=214, Bw=4,
RefWavelength=Off,
RefBandwidth=Off,
S1=Auto, Avg=On,
%B: 0,0%
Flow 0,500 ml/min
monomeric rHA
2-GM-CSF-33.292
1-22,791
min

FIG. 7

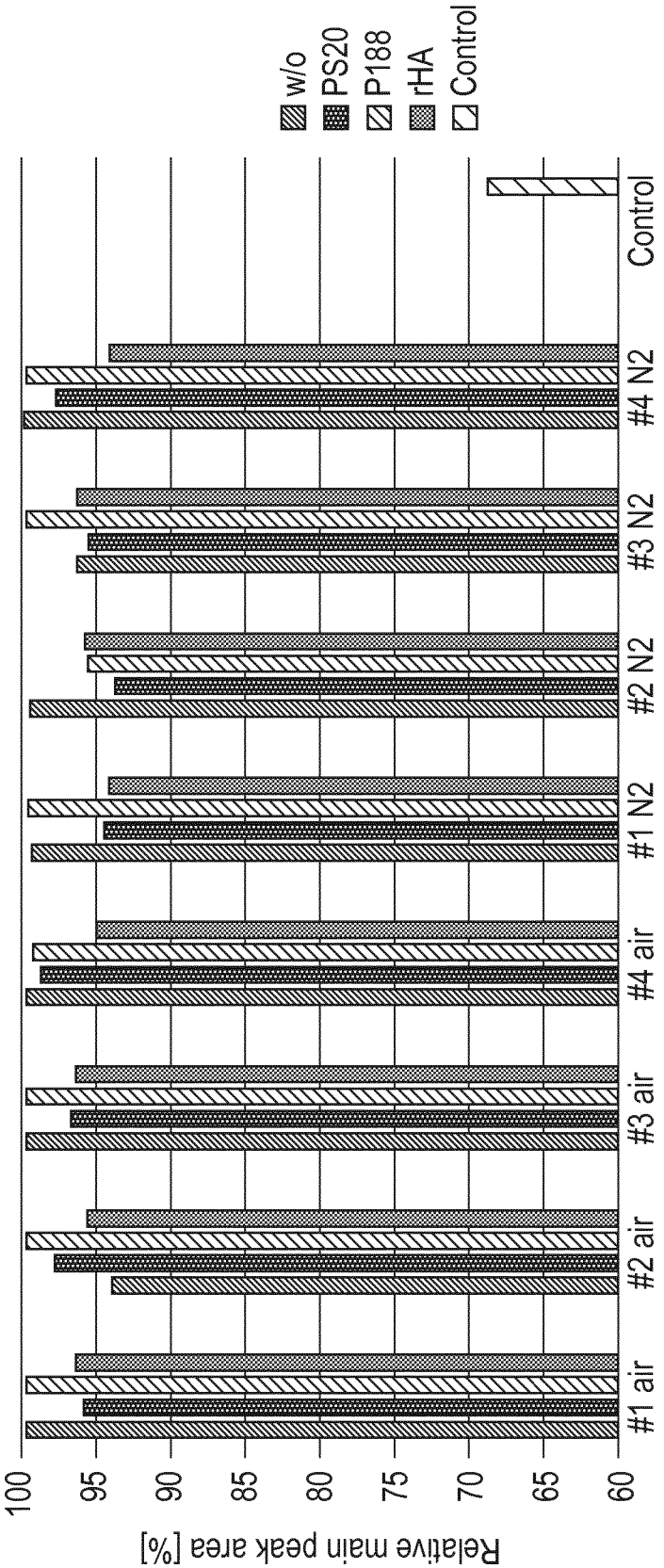
w/o
PS20
P188
rHA
Control
Relative main peak area [%]
100
95
90
85
80
75
70
65
60
#1 air
#2 air
#3 air
#4 air
#1 N2
#2 N2
#3 N2
#4 N2
Control
Control Formulation Variants

FIG. 8

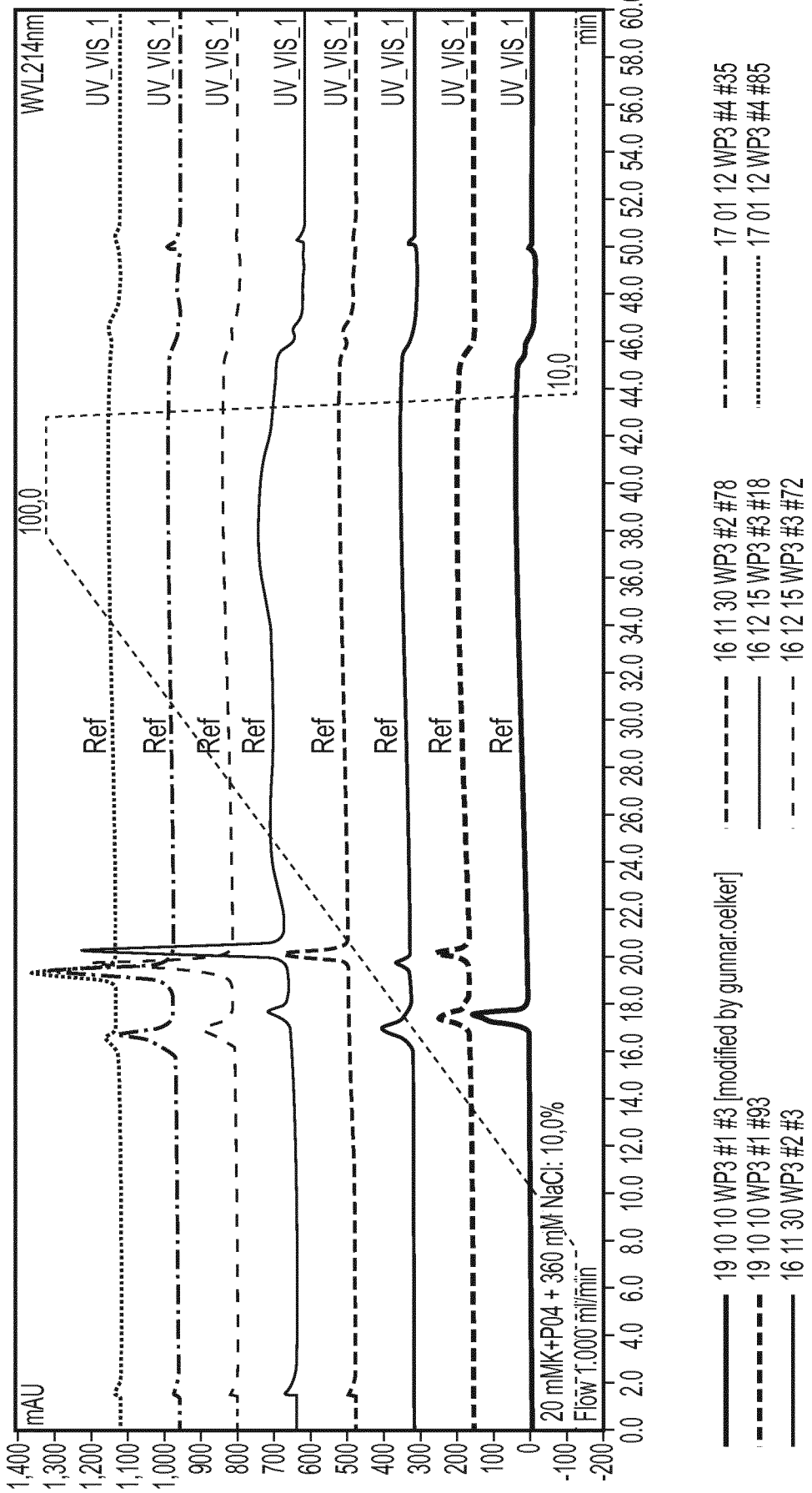
mAU
WVL214nm
UV_VIS_1
Ref
100,0
10,0
min
20 mMK+P04 + 360 mM NaCl: 10,0%
Flow 1.000 ml/min
19 10 10 WP3 #1 #3 [modified by gunnar.oelker]
19 10 10 WP3 #1 #93
16 11 30 WP3 #2 #3
16 11 30 WP3 #2 #78
16 12 15 WP3 #3 #18
16 12 15 WP3 #3 #72
17 01 12 WP3 #4 #35
17 01 12 WP3 #4 #85

FIG. 9

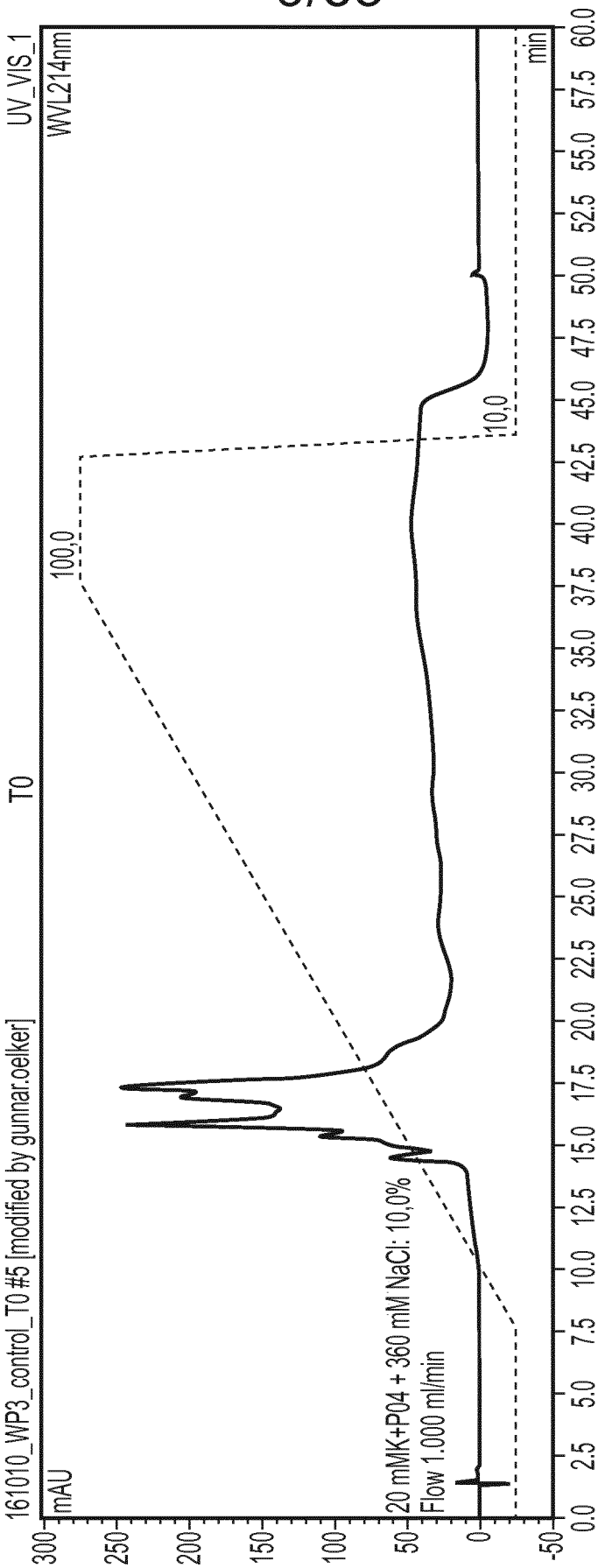
161010_WP3_control_T0 #5 [modified by gunnar.oelker]
T0
UV_VIS_1
WVL214nm
mAU
min
100,0
10,0
20 mMK+P04 + 360 mM NaCl: 10,0%
Flow 1.000 ml/min
300
250
200
150
100
50
0
-50
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
52.5
55.0
57.5
60.0

FIG. 10

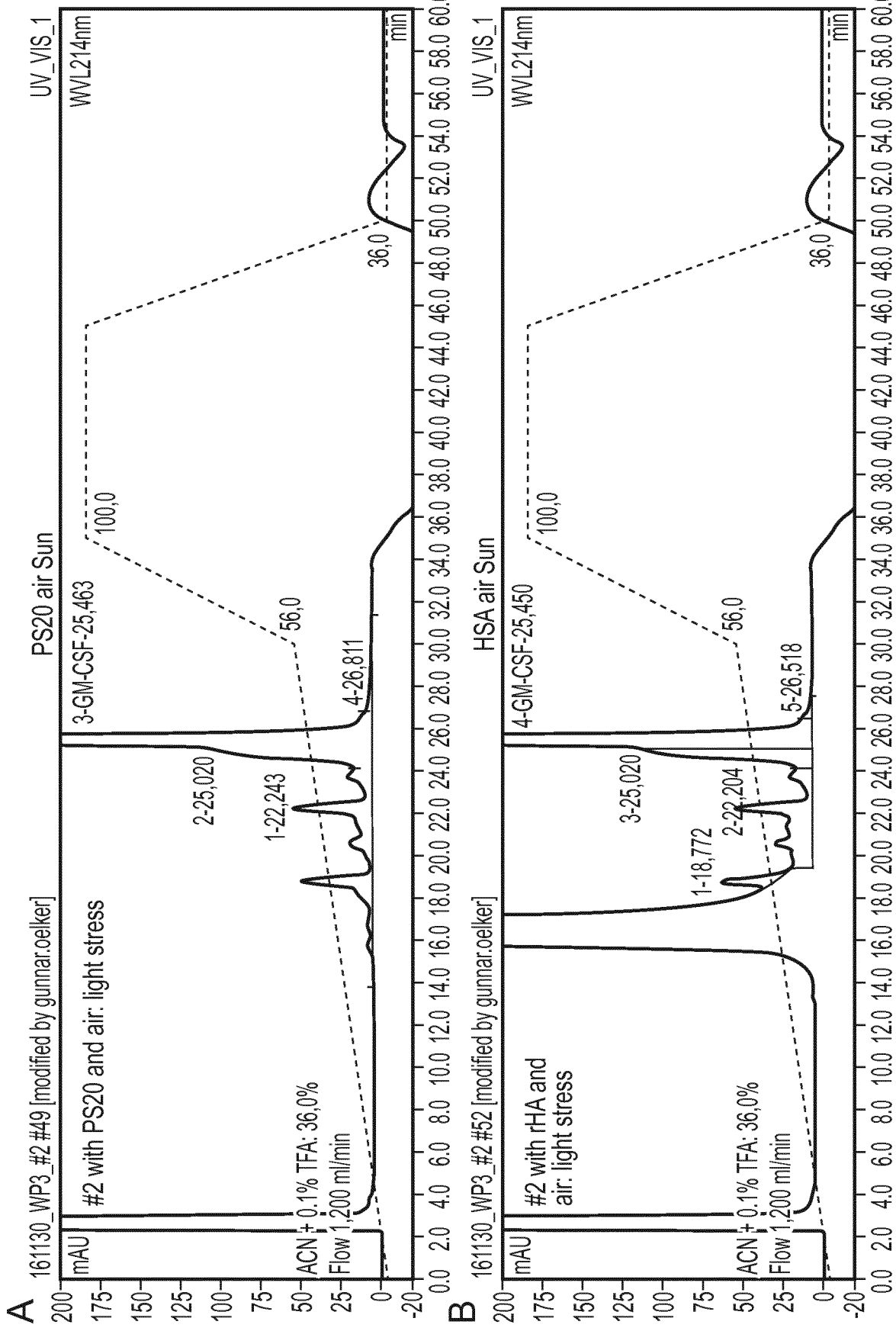
A
161130_WP3_#2 #49 [modified by gunnar.oelker]
PS20 air Sun
UV_VIS_1
WVL214nm
mAU
#2 with PS20 and air: light stress
3-GM-CSF-25,463
100,0
56,0
2-25,020
1-22,243
4-26,811
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
min
B
161130_WP3_#2 #52 [modified by gunnar.oelker]
HSA air Sun
UV_VIS_1
WVL214nm
mAU
#2 with rHA and air: light stress
4-GM-CSF-25,450
100,0
56,0
3-25,020
1-18,772
2-22,204
5-26,518
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
min

FIG. 11

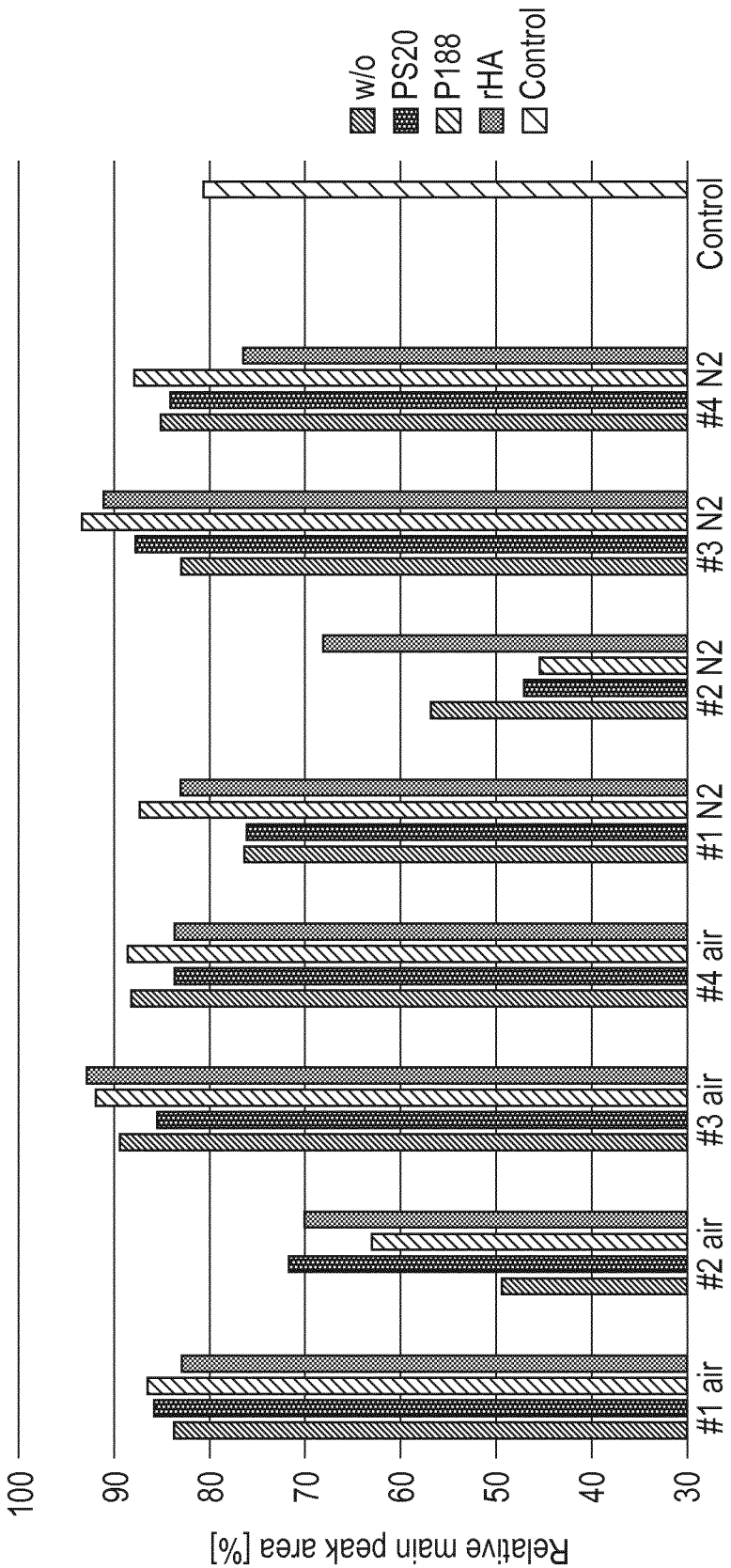
w/o
PS20
P188
rHA
Control
100
90
80
70
60
50
40
30
Relative main peak area [%]
#1 air
#2 air
#3 air
#4 air
#1 N2
#2 N2
#3 N2
#4 N2
Control
Formulation variant

FIG. 12

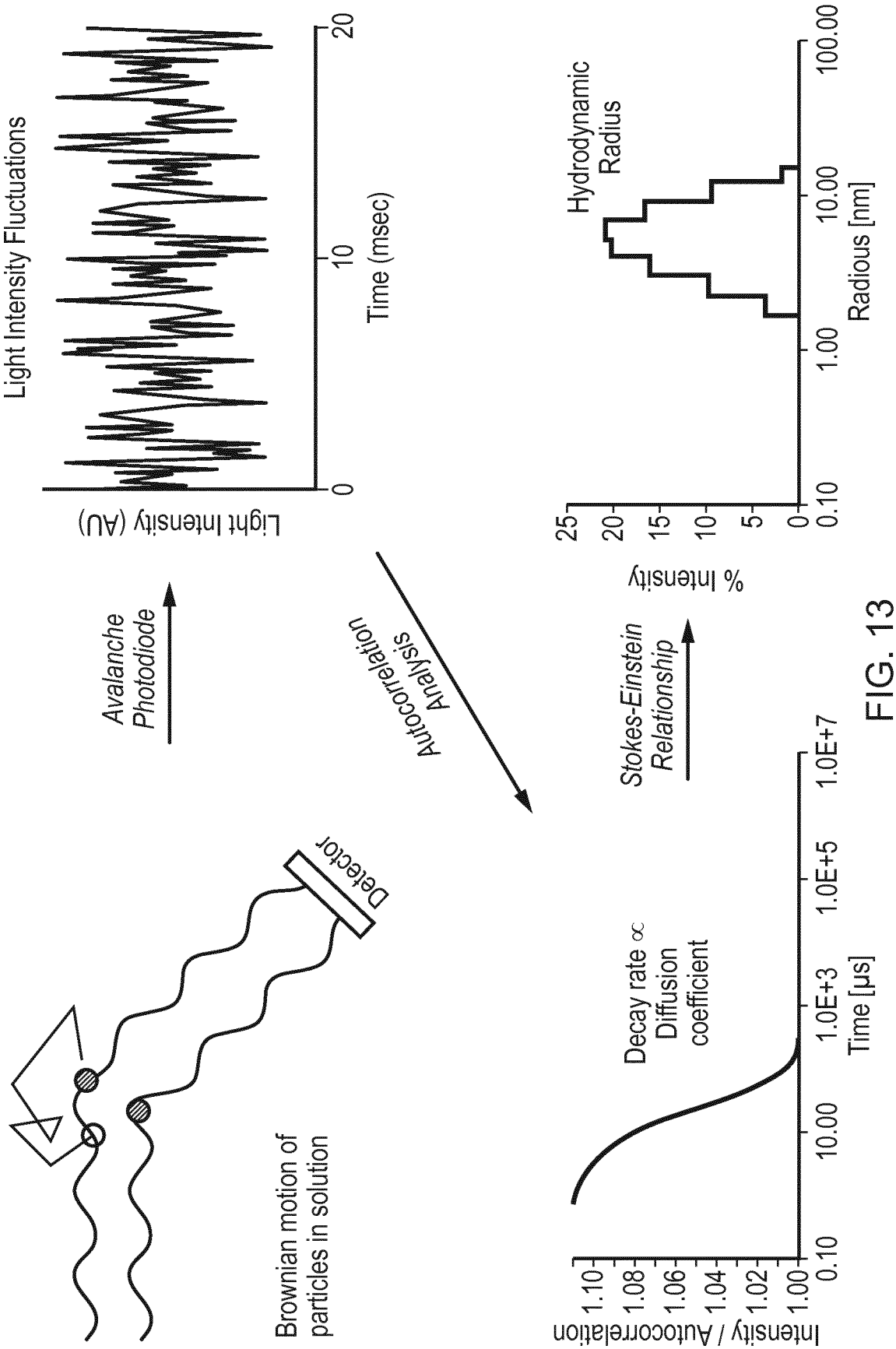
Light Intensity Fluctuations
Light Intensity (AU)
Time (msec)
0
10
20
Avalanche Photodiode
Autocorrelation Analysis
Hydrodynamic Radius
% Intensity
Radious [nm]
0.10
1.00
10.00
100.00
Detector
Brownian motion of particles in solution
Stokes-Einstein Relationship
Decay rate ∝ Diffusion coefficient
Intensity / Autocorrelation
Time [µs]
1.00
1.02
1.04
1.06
1.08
1.10
1.0E+3
1.0E+5
1.0E+7

FIG. 13

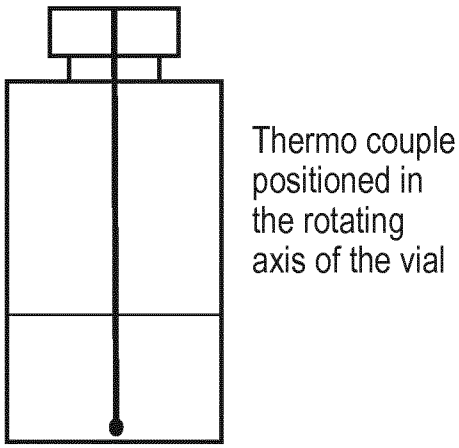
Thermo couple
positioned in
the rotating
axis of the vial

FIG. 14

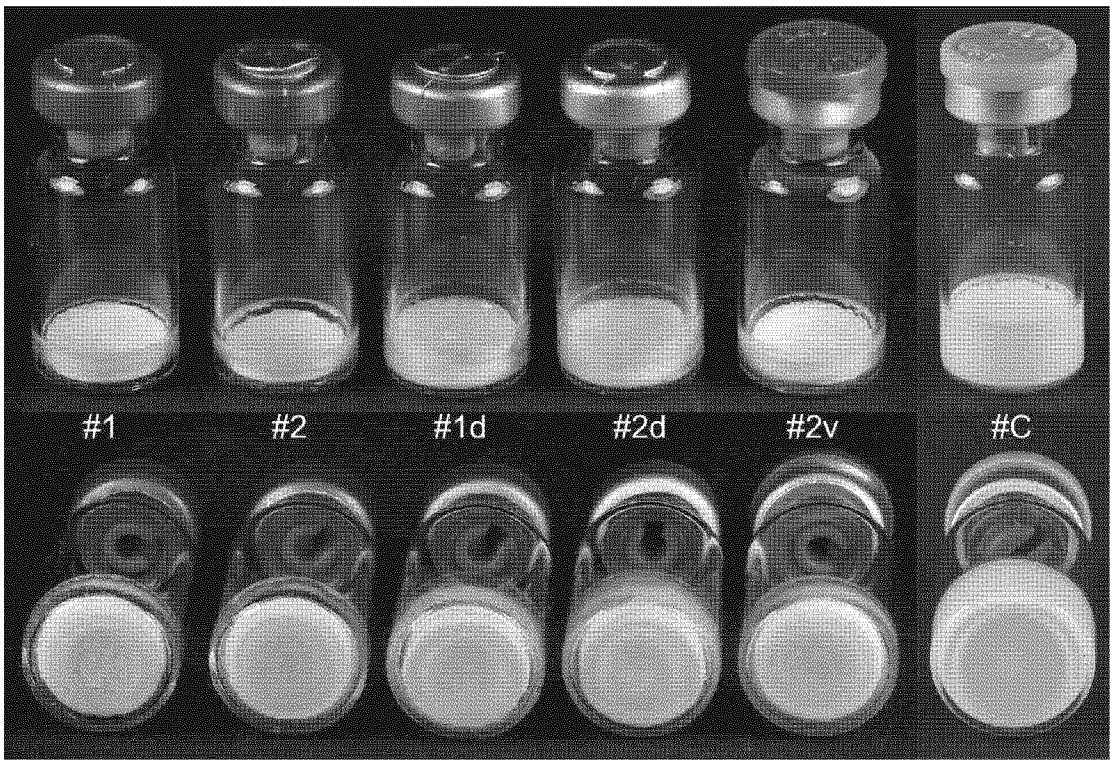
#1
#2
#1d
#2d
#2v
#C

FIG. 15

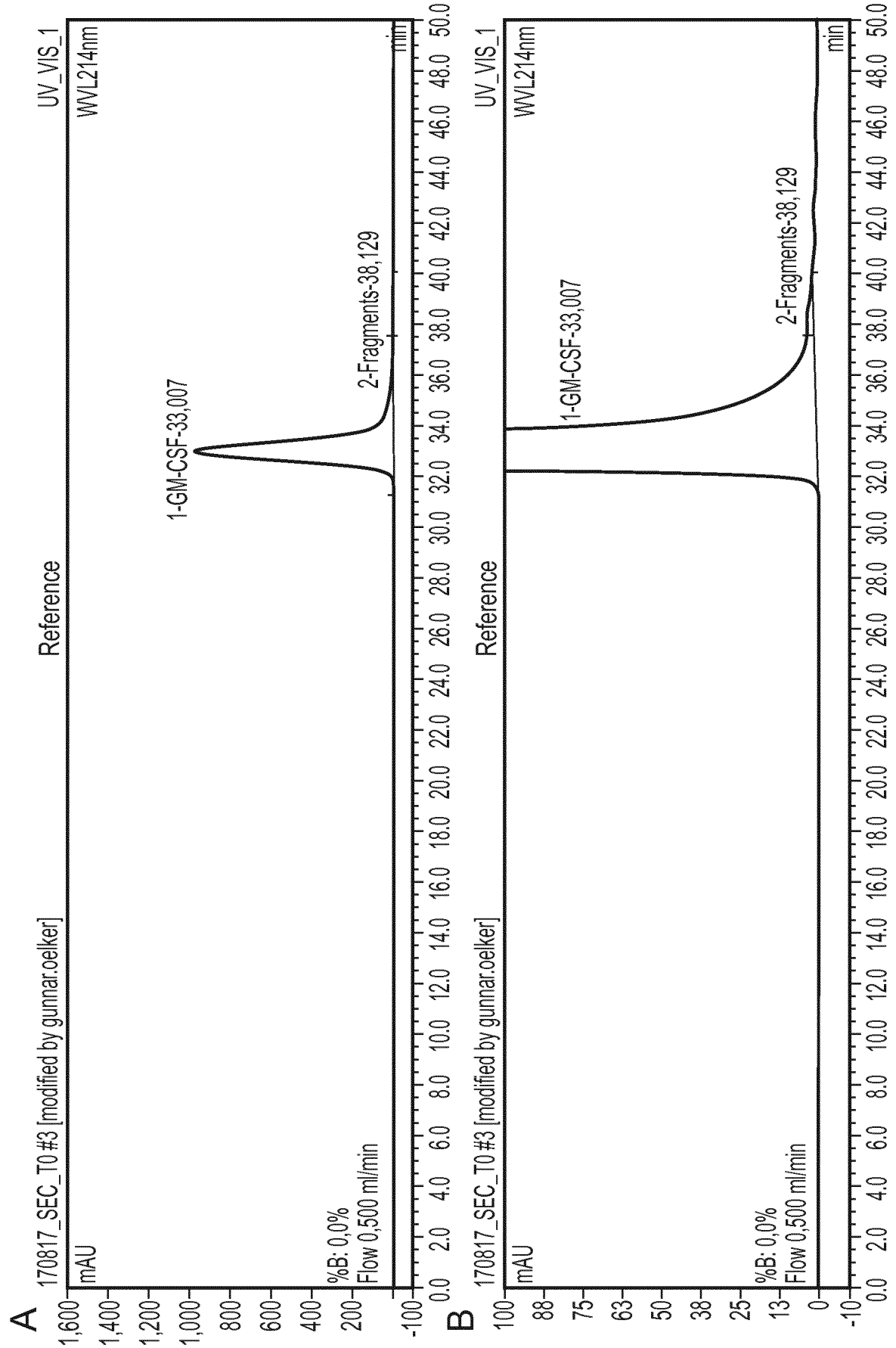
A
170817_SEC_T0 #3 [modified by gunnar.oelker]
Reference
UV_VIS_1
WVL214nm
mAU
1-GM-CSF-33,007
2-Fragments-38,129
%B: 0,0%
Flow 0,500 ml/min
min
B
170817_SEC_T0 #3 [modified by gunnar.oelker]
Reference
UV_VIS_1
WVL214nm
mAU
1-GM-CSF-33,007
2-Fragments-38,129
%B: 0,0%
Flow 0,500 ml/min
min

FIG. 16

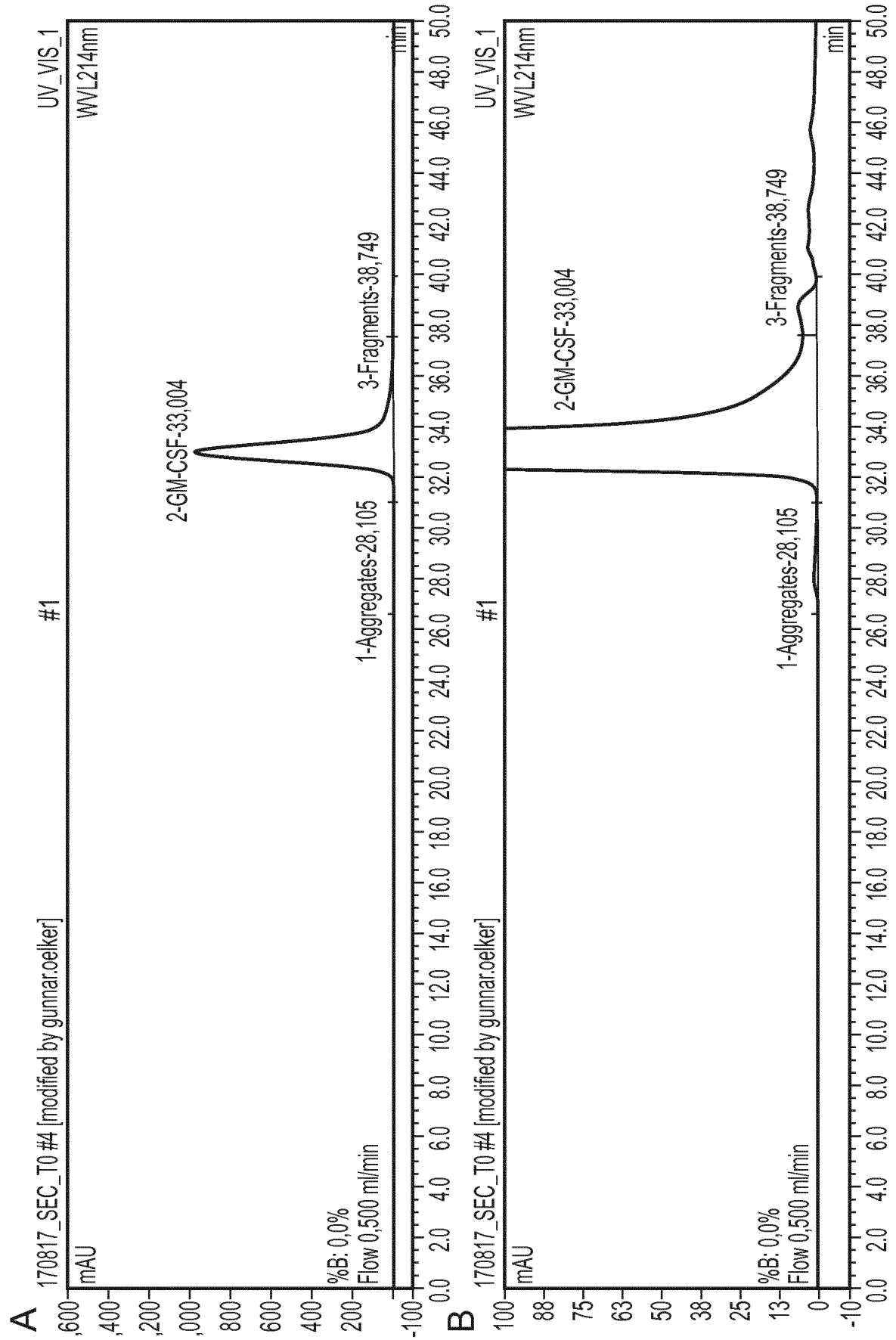
A
170817_SEC_T0 #4 [modified by gunnar.oelker]
#1
UV_VIS_1
WVL214nm
mAU
%B: 0,0%
Flow 0,500 ml/min
2-GM-CSF-33,004
1-Aggregates-28,105
3-Fragments-38,749
min
B
170817_SEC_T0 #4 [modified by gunnar.oelker]
#1
UV_VIS_1
WVL214nm
mAU
%B: 0,0%
Flow 0,500 ml/min
2-GM-CSF-33,004
1-Aggregates-28,105
3-Fragments-38,749
min

FIG. 17

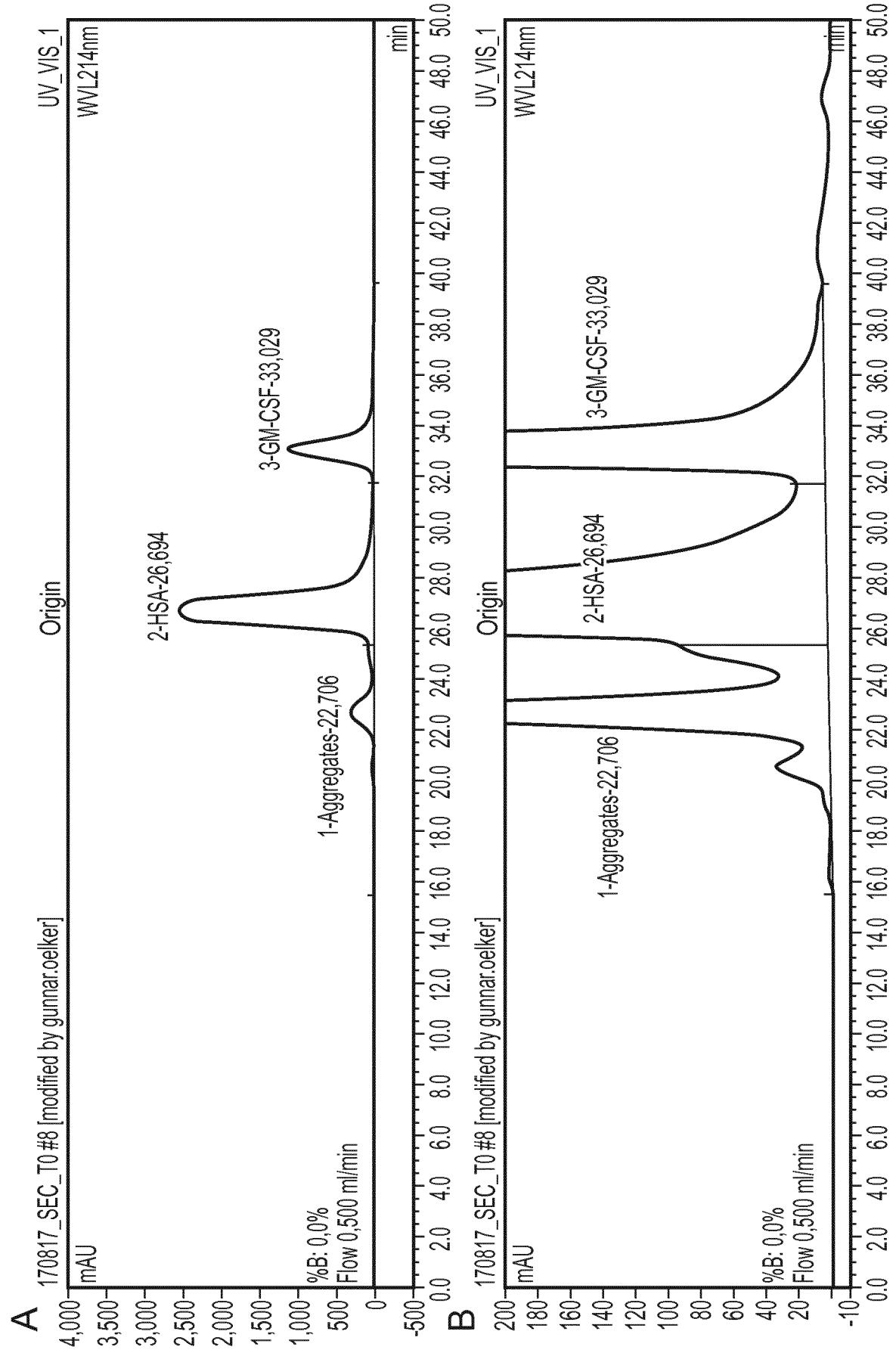
A
170817_SEC_T0 #8 [modified by gunnar.oelker]
Origin
UV_VIS_1
WVL214nm
mAU
%B: 0,0%
Flow 0,500 ml/min
1-Aggregates-22,706
2-HSA-26,694
3-GM-CSF-33,029
min
B
170817_SEC_T0 #8 [modified by gunnar.oelker]
Origin
UV_VIS_1
WVL214nm
mAU
%B: 0,0%
Flow 0,500 ml/min
1-Aggregates-22,706
2-HSA-26,694
3-GM-CSF-33,029
min

FIG. 18

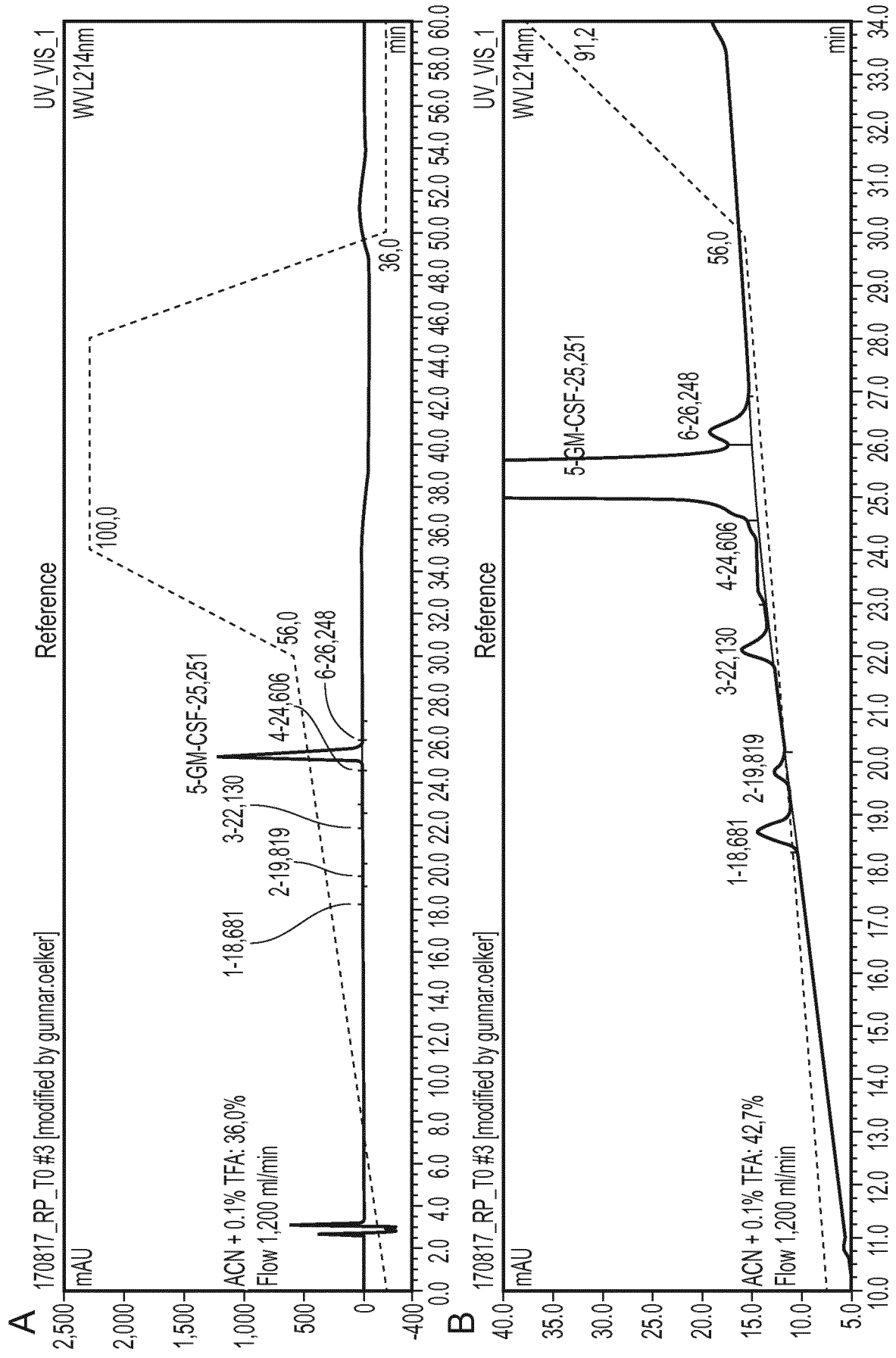
A
170817_RP_T0 #3 [modified by gunnar.oelker]
Reference
UV_VIS_1
WVL214nm
mAU
min
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
100,0
36,0
1-18,681
2-19,819
3-22,130
5-GM-CSF-25,251
4-24,606
56,0
6-26,248
B
170817_RP_T0 #3 [modified by gunnar.oelker]
Reference
UV_VIS_1
WVL214nm
mAU
min
ACN + 0.1% TFA: 42,7%
Flow 1,200 ml/min
91,2
56,0
1-18,681
2-19,819
3-22,130
4-24,606
5-GM-CSF-25,251
6-26,248

FIG. 19

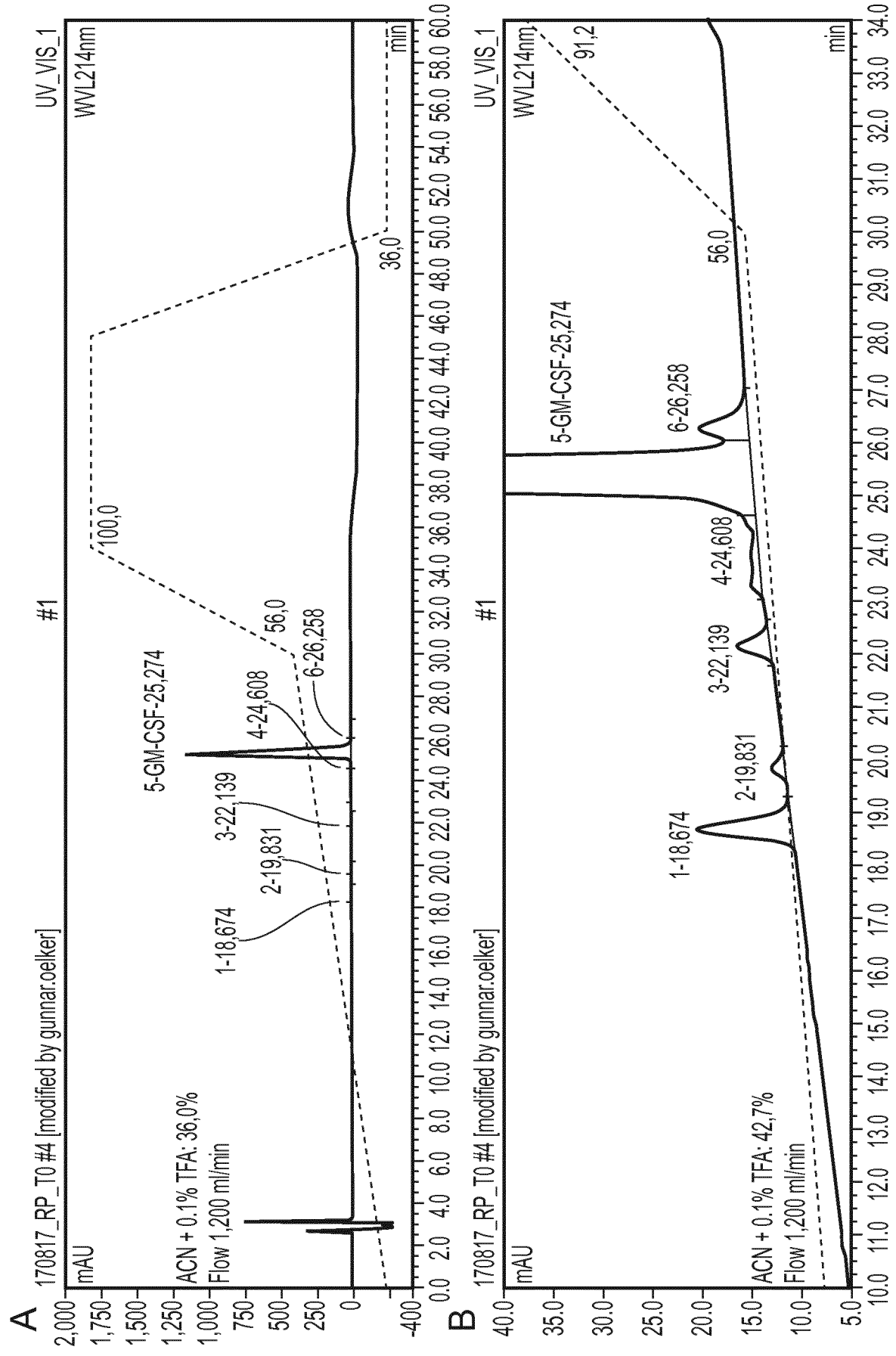
A
170817_RP_T0 #4 [modified by gunnar.oelker]
#1
UV_VIS_1
WVL214nm
mAU
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
100,0
36,0
56,0
5-GM-CSF-25,274
1-18,674
2-19,831
3-22,139
4-24,608
6-26,258
min
B
170817_RP_T0 #4 [modified by gunnar.oelker]
#1
UV_VIS_1
WVL214nm
mAU
ACN + 0.1% TFA: 42,7%
Flow 1,200 ml/min
91,2
56,0
5-GM-CSF-25,274
1-18,674
2-19,831
3-22,139
4-24,608
6-26,258
min

FIG. 20

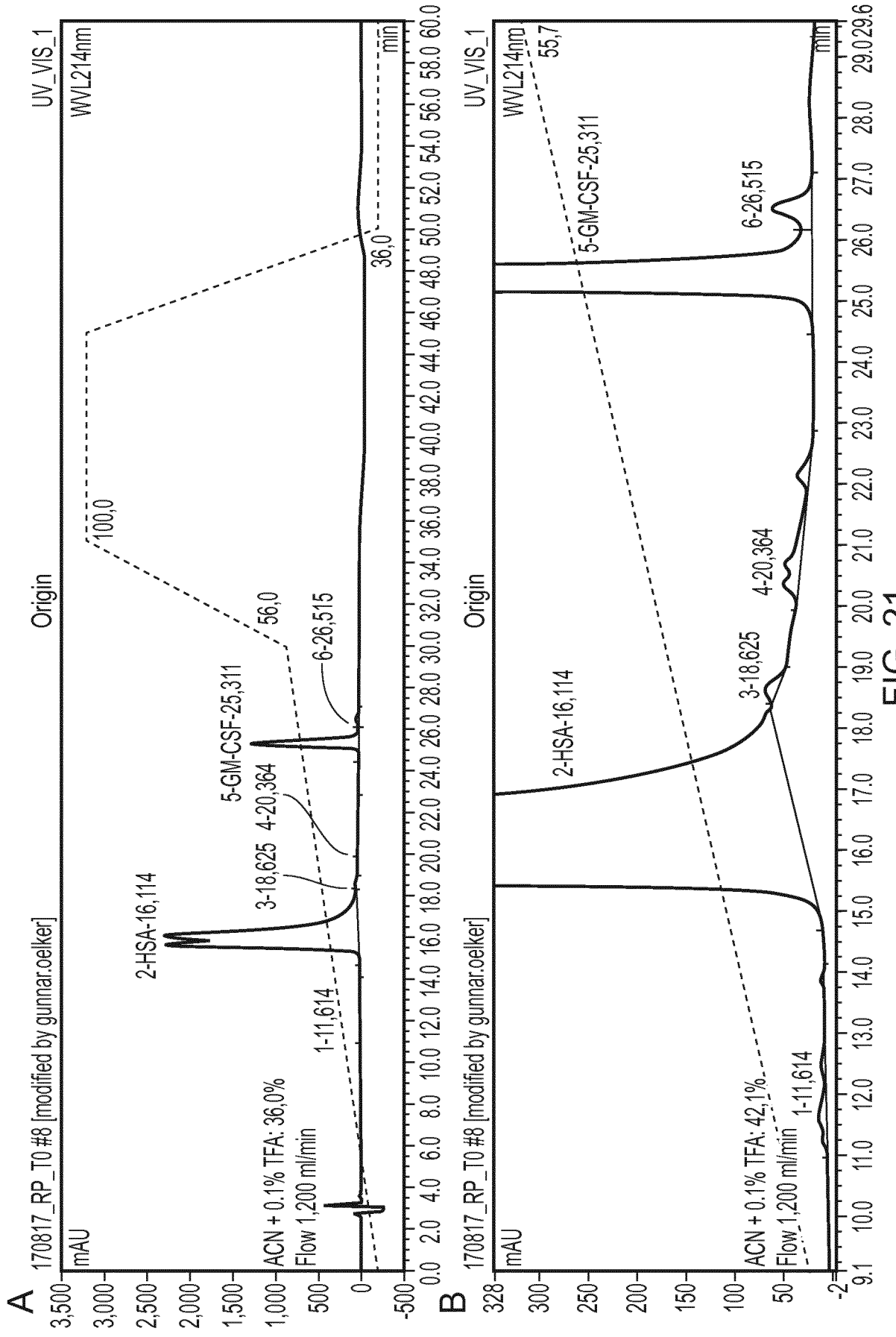
A
170817_RP_T0 #8 [modified by gunnar.oelker]
UV_VIS_1
WVL214nm
mAU
Origin
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
100,0
56,0
36,0
1-11,614
2-HSA-16,114
3-18,625
4-20,364
5-GM-CSF-25,311
6-26,515
min
B
170817_RP_T0 #8 [modified by gunnar.oelker]
UV_VIS_1
WVL214nm
mAU
Origin
ACN + 0.1% TFA: 42,1%
Flow 1,200 ml/min
55,7
1-11,614
2-HSA-16,114
3-18,625
4-20,364
5-GM-CSF-25,311
6-26,515
min

FIG. 21

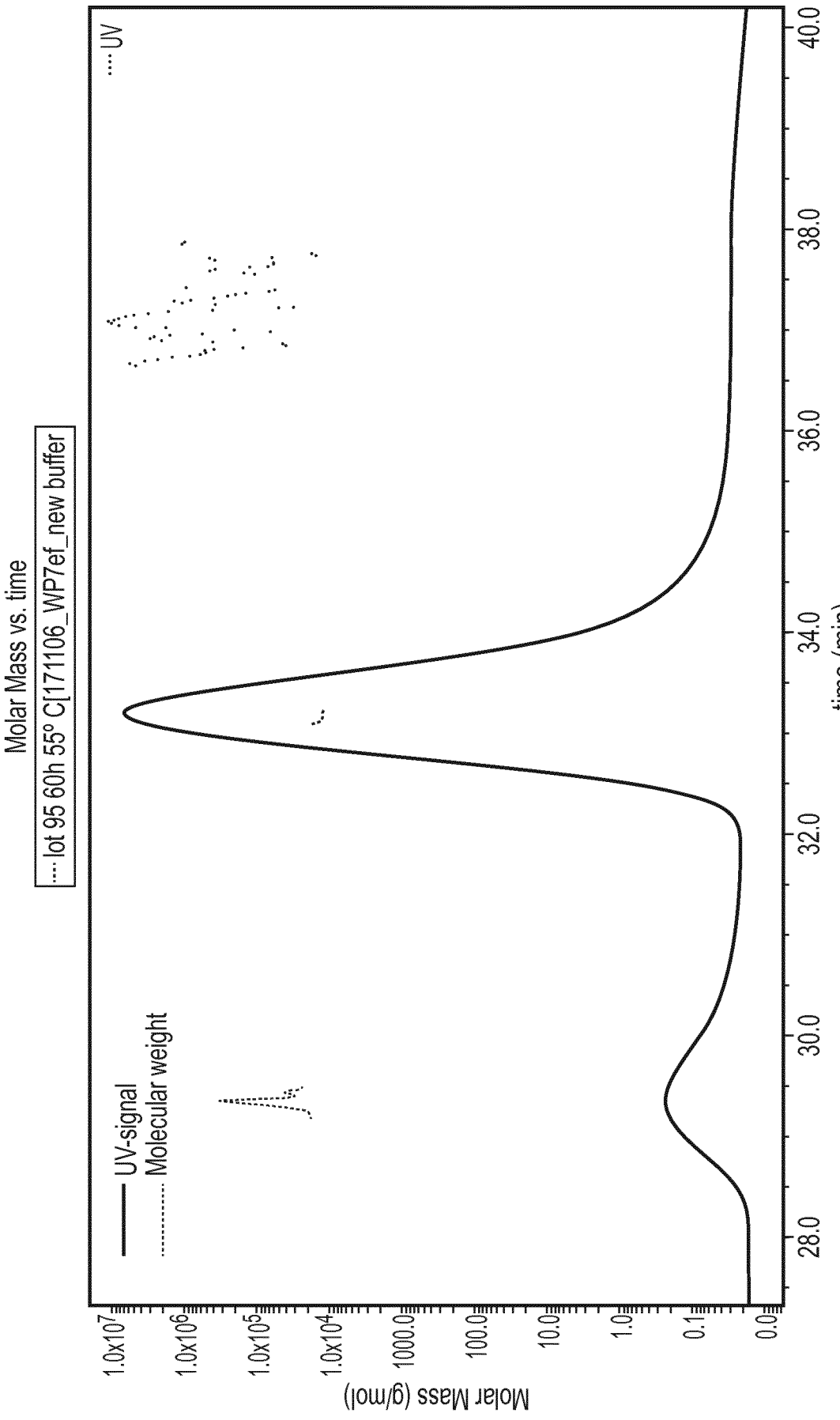
Molar Mass vs. time
lot 95 60h 55° C[171106_WP7ef_new buffer
UV
UV-signal
Molecular weight
Molar Mass (g/mol)
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
1000.0
100.0
10.0
1.0
0.1
0.0
28.0
30.0
32.0
34.0
36.0
38.0
40.0
time (min)

FIG. 22

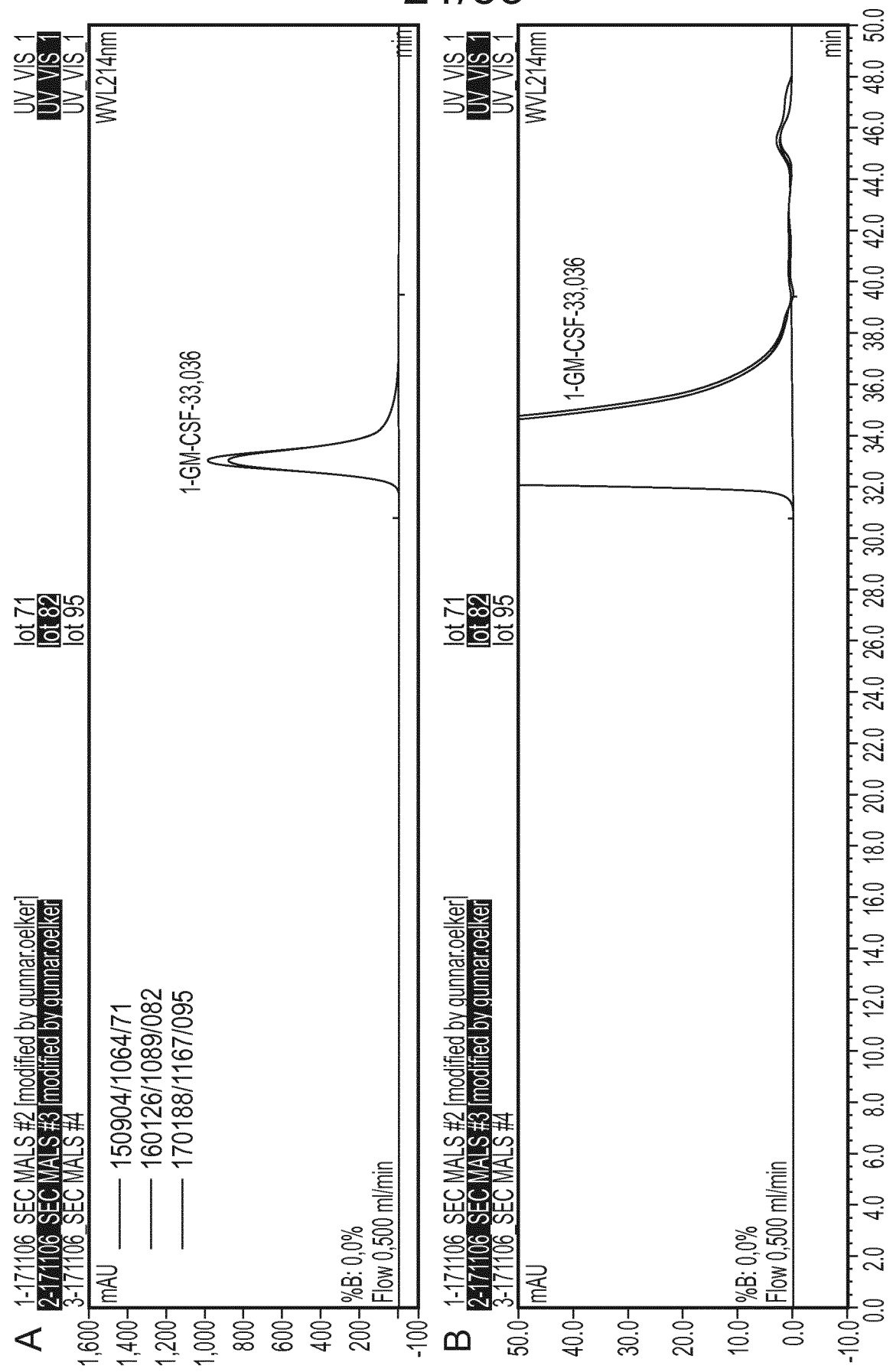
A
1-171106_SEC MALS #2 [modified by gunnar.oelker]
2-171106_SEC MALS #3 [modified by gunnar.oelker]
3-171106_SEC MALS #4
lot 71
lot 82
lot 95
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
150904/1064/71
160126/1089/082
170188/1167/095
1-GM-CSF-33,036
%B: 0,0%
Flow 0,500 ml/min
min
B
1-171106_SEC MALS #2 [modified by gunnar.oelker]
2-171106_SEC MALS #3 [modified by gunnar.oelker]
3-171106_SEC MALS #4
lot 71
lot 82
lot 95
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
1-GM-CSF-33,036
%B: 0,0%
Flow 0,500 ml/min
min

FIG. 23

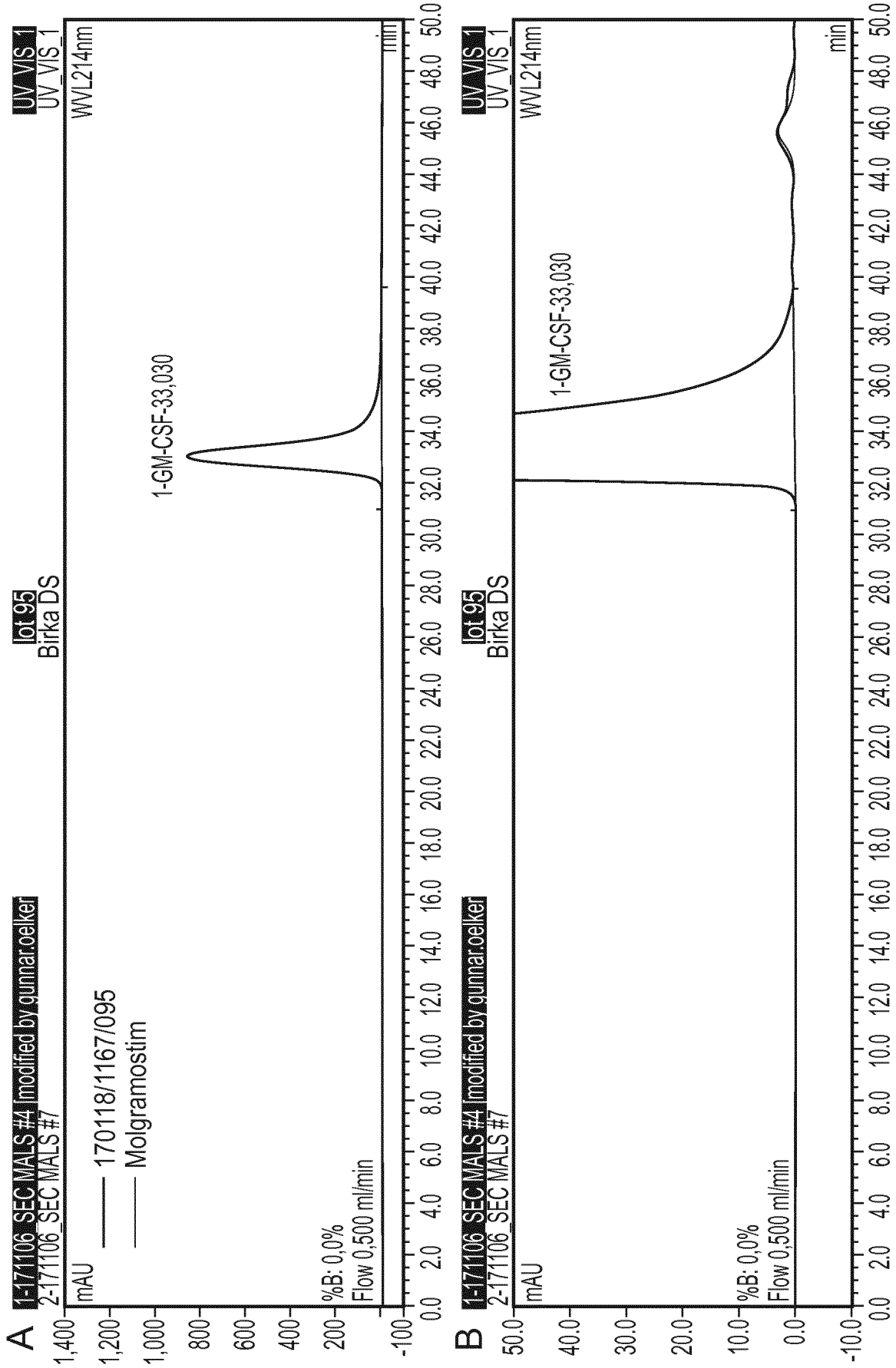
A
1-171106_SEC MALS #4 modified by gunnar.oelker
2-171106_SEC MALS #7
lot 95
Birka DS
UV_VIS_1
WVL214nm
mAU
170118/1167/095
Molgramostim
1-GM-CSF-33,030
%B: 0,0%
Flow 0,500 ml/min
min
B
1-171106_SEC MALS #4 modified by gunnar.oelker
2-171106_SEC MALS #7
lot 95
Birka DS
UV_VIS_1
WVL214nm
mAU
1-GM-CSF-33,030
%B: 0,0%
Flow 0,500 ml/min
min

FIG. 24

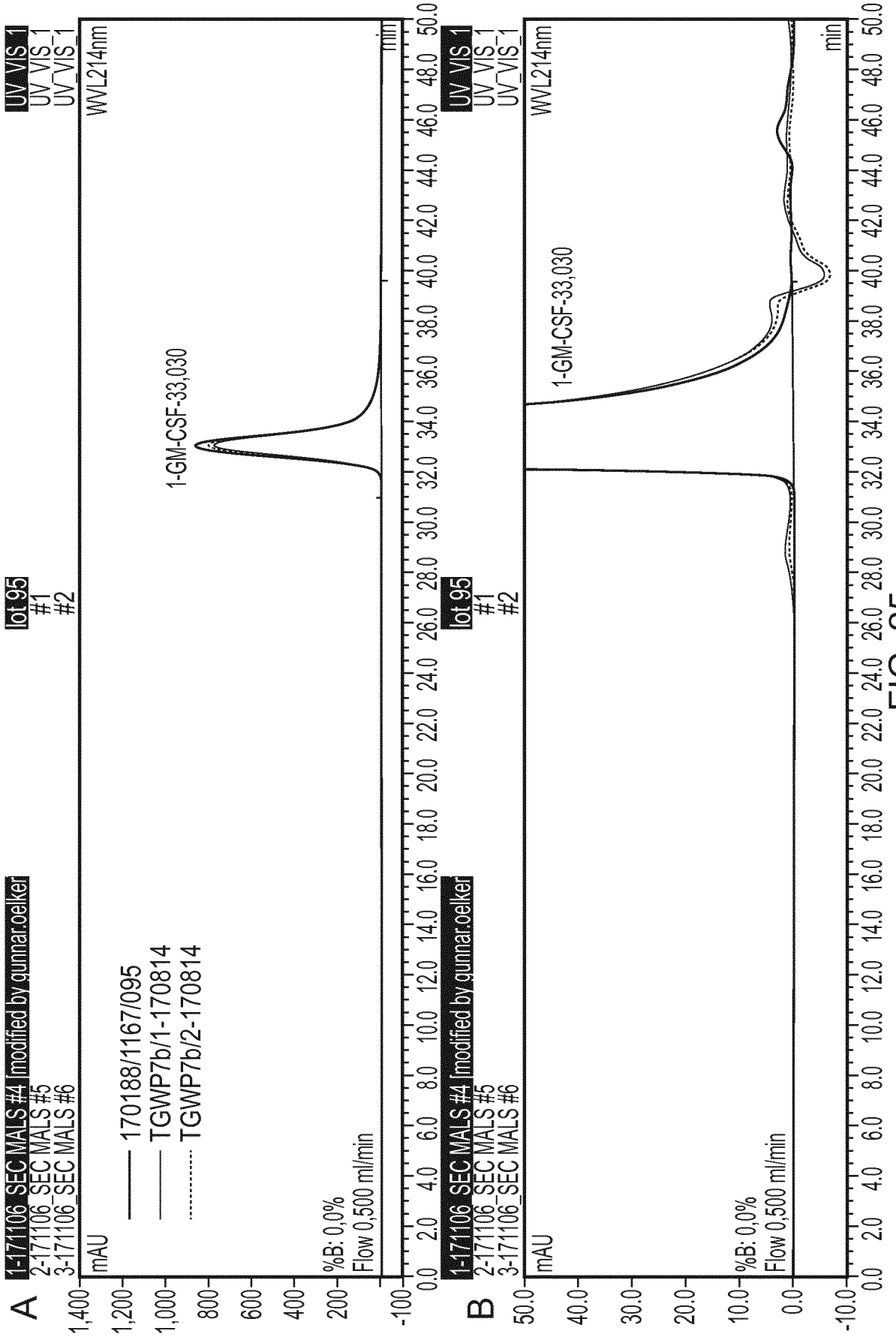
A
1-171106_SEC MALS #4 [modified by gunnar.oelker]
2-171106_SEC MALS #5
3-171106_SEC MALS #6
lot 95
#1
#2
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
170188/1167/095
TGWP7b/1-170814
TGWP7b/2-170814
1-GM-CSF-33,030
%B: 0,0%
Flow 0,500 ml/min
min
B
1-171106_SEC MALS #4 [modified by gunnar.oelker]
2-171106_SEC MALS #5
3-171106_SEC MALS #6
lot 95
#1
#2
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
1-GM-CSF-33,030
%B: 0,0%
Flow 0,500 ml/min
min

FIG. 25

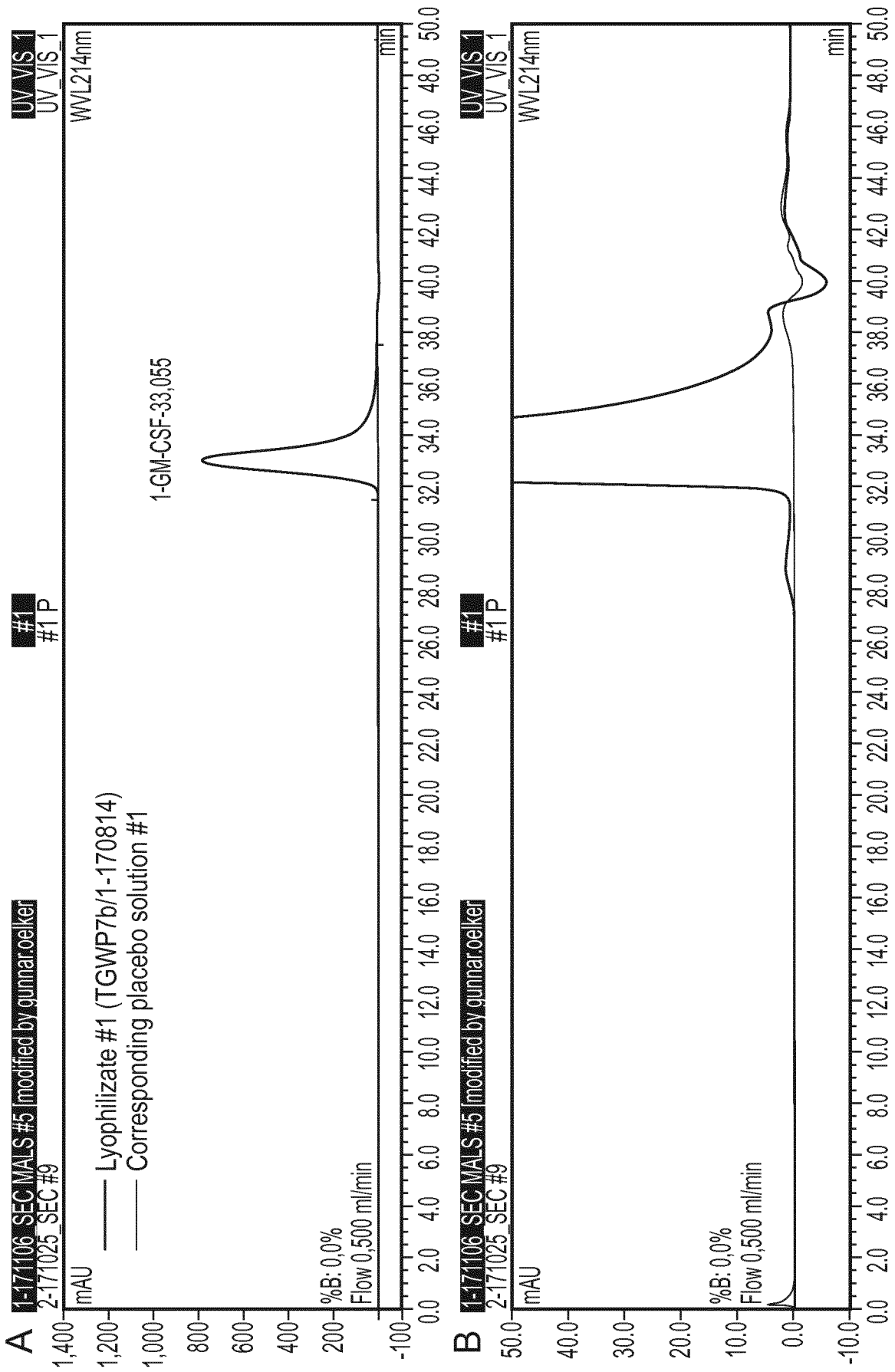
A
1-171106_SEC MALS #5 [modified by gunnar.oelker]
2-171025_SEC #9
#1
#1 P
UV_VIS_1
UV_VIS_1
WVL:214nm
mAU
Lyophilizate #1 (TGWP7b/1-170814)
Corresponding placebo solution #1
1-GM-CSF-33,055
%B: 0,0%
Flow 0,500 ml/min
min
B
1-171106_SEC MALS #5 [modified by gunnar.oelker]
2-171025_SEC #9
#1
#1 P
UV_VIS_1
UV_VIS_1
WVL:214nm
mAU
%B: 0,0%
Flow 0,500 ml/min
min

FIG. 26

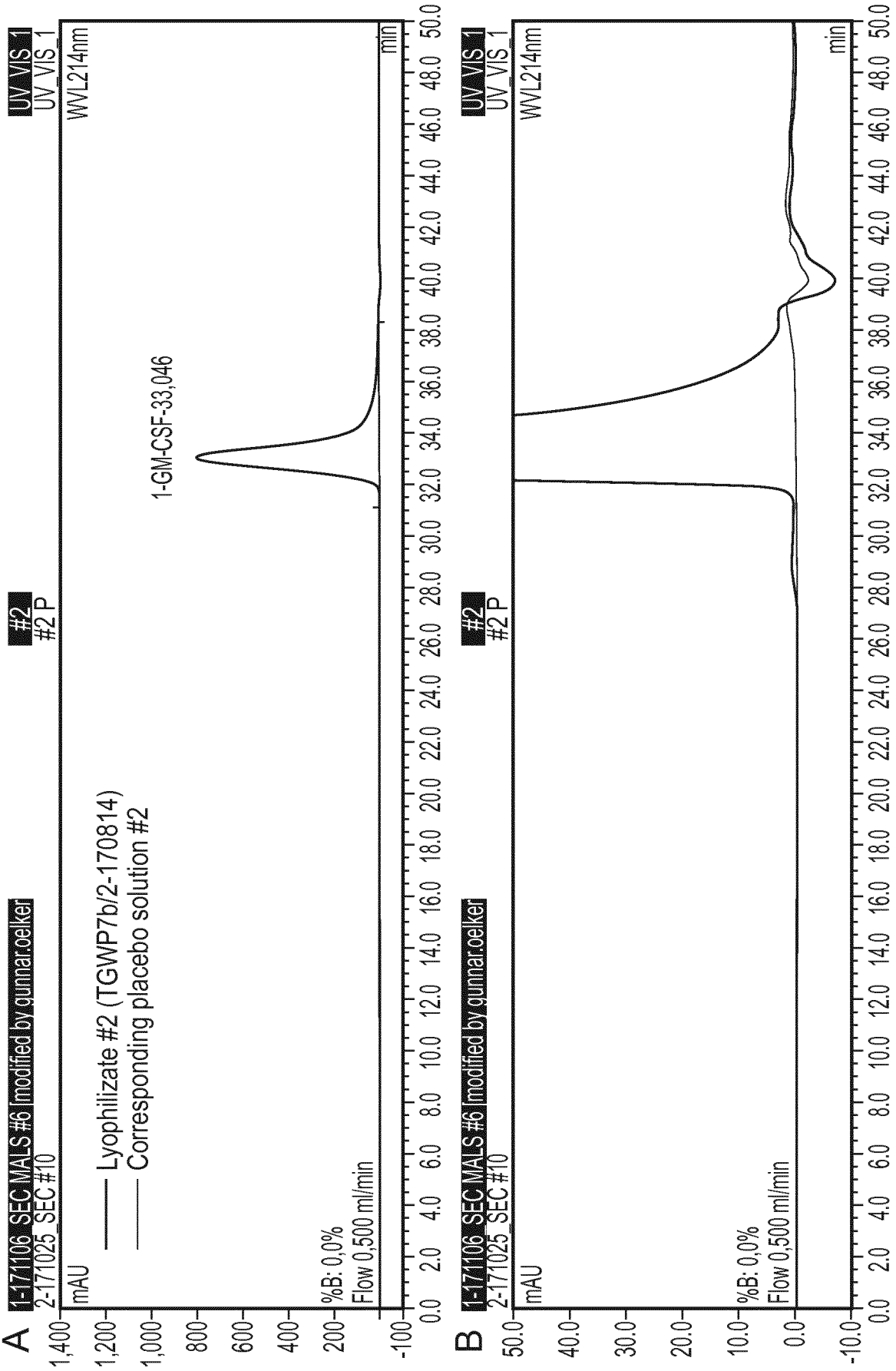
A
1-171106 SEC MALS #6 [modified by cunnar.oelker]
2-171025_SEC #10
#2
#2 P
UV VIS 1
UV_VIS_1
WVL214nm
mAU
Lyophilizate #2 (TGWP7b/2-170814)
Corresponding placebo solution #2
1-GM-CSF-33,046
%B: 0,0%
Flow 0,500 ml/min
min
B
1-171106 SEC MALS #6 [modified by cunnar.oelker]
2-171025_SEC #10
#2
#2 P
UV VIS 1
UV_VIS_1
WVL214nm
mAU
%B: 0,0%
Flow 0,500 ml/min
min

FIG. 27

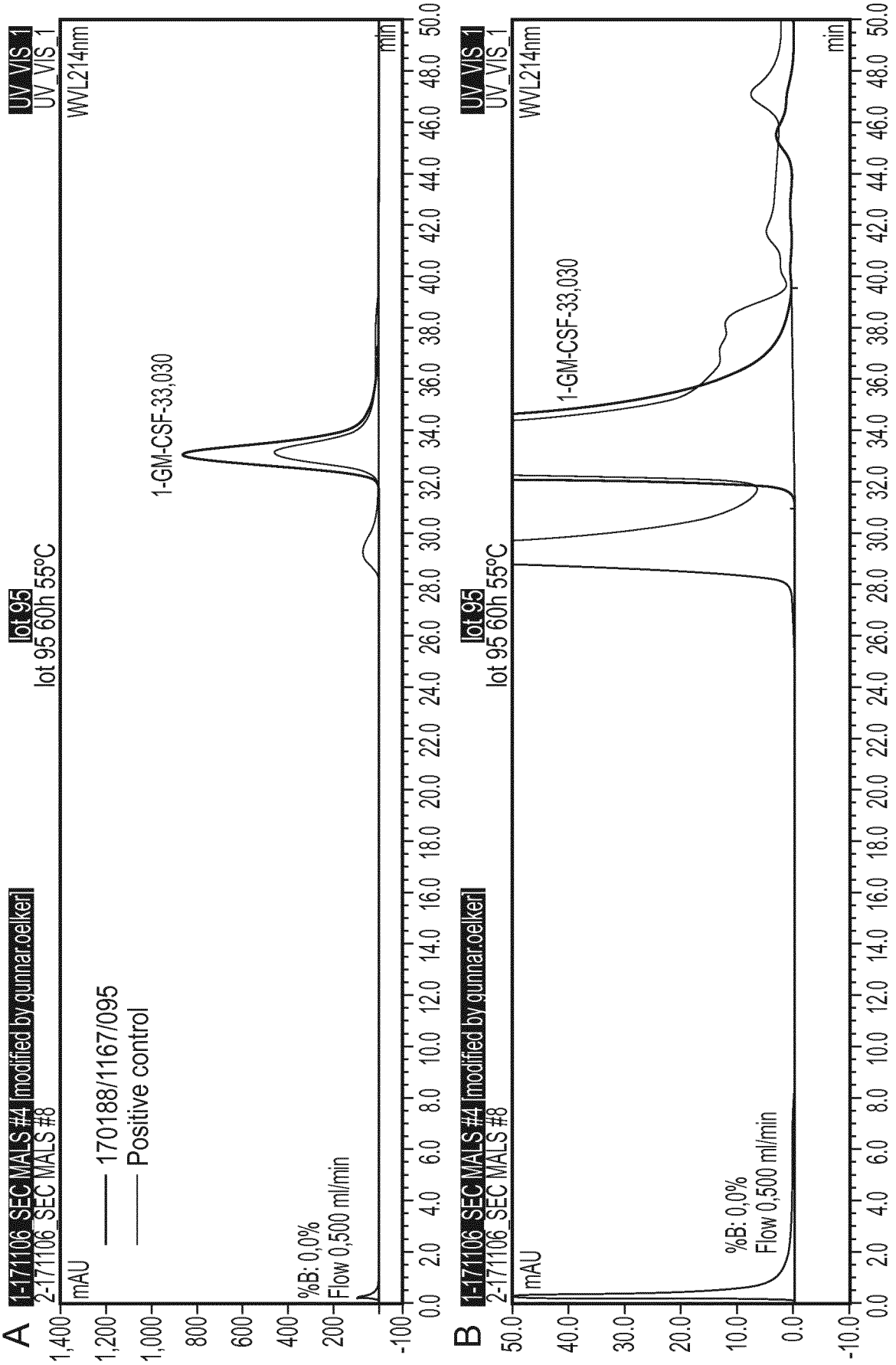
A
1-171106_SEC MALS #4 modified by gunnar.oelkerl
2-171106_SEC MALS #8
lot 95
lot 95 60h 55°C
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
170188/1167/095
Positive control
1-GM-CSF-33,030
%B: 0,0%
Flow 0,500 ml/min
min
B
1-171106_SEC MALS #4 modified by gunnar.oelkerl
2-171106_SEC MALS #8
lot 95
lot 95 60h 55°C
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
1-GM-CSF-33,030
%B: 0,0%
Flow 0,500 ml/min
min

FIG. 28

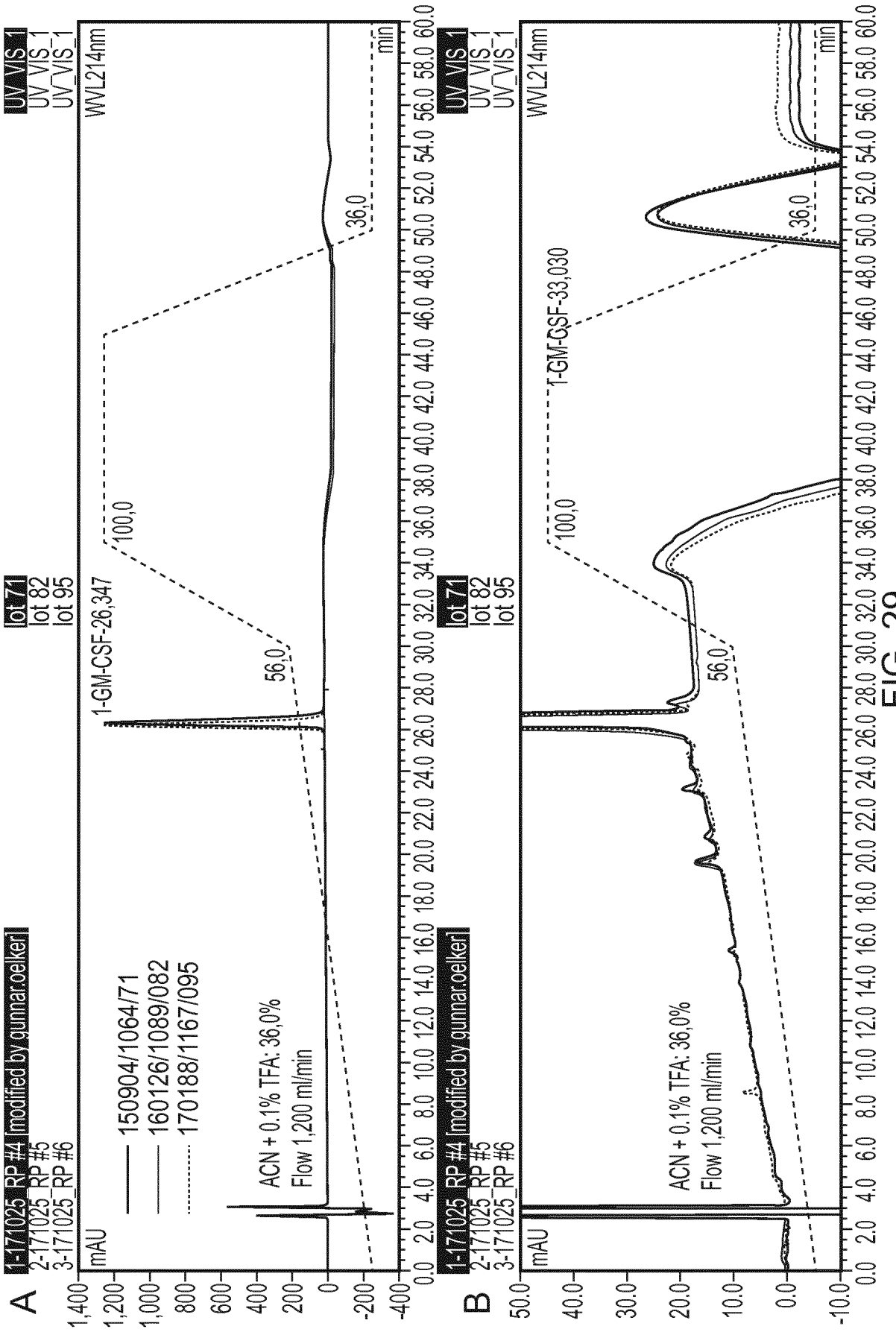
A
B
1-171025_RP #4 modified by gunnar.oelkerl
2-171025_RP #5
3-171025_RP #6
mAU
150904/1064/71
160126/1089/082
170188/1167/095
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
1-GM-CSF-26,347
1-GM-CSF-33,030
100,0
56,0
36,0
lot 71
lot 82
lot 95
UV_VIS_1
WVL214nm
min

FIG. 29

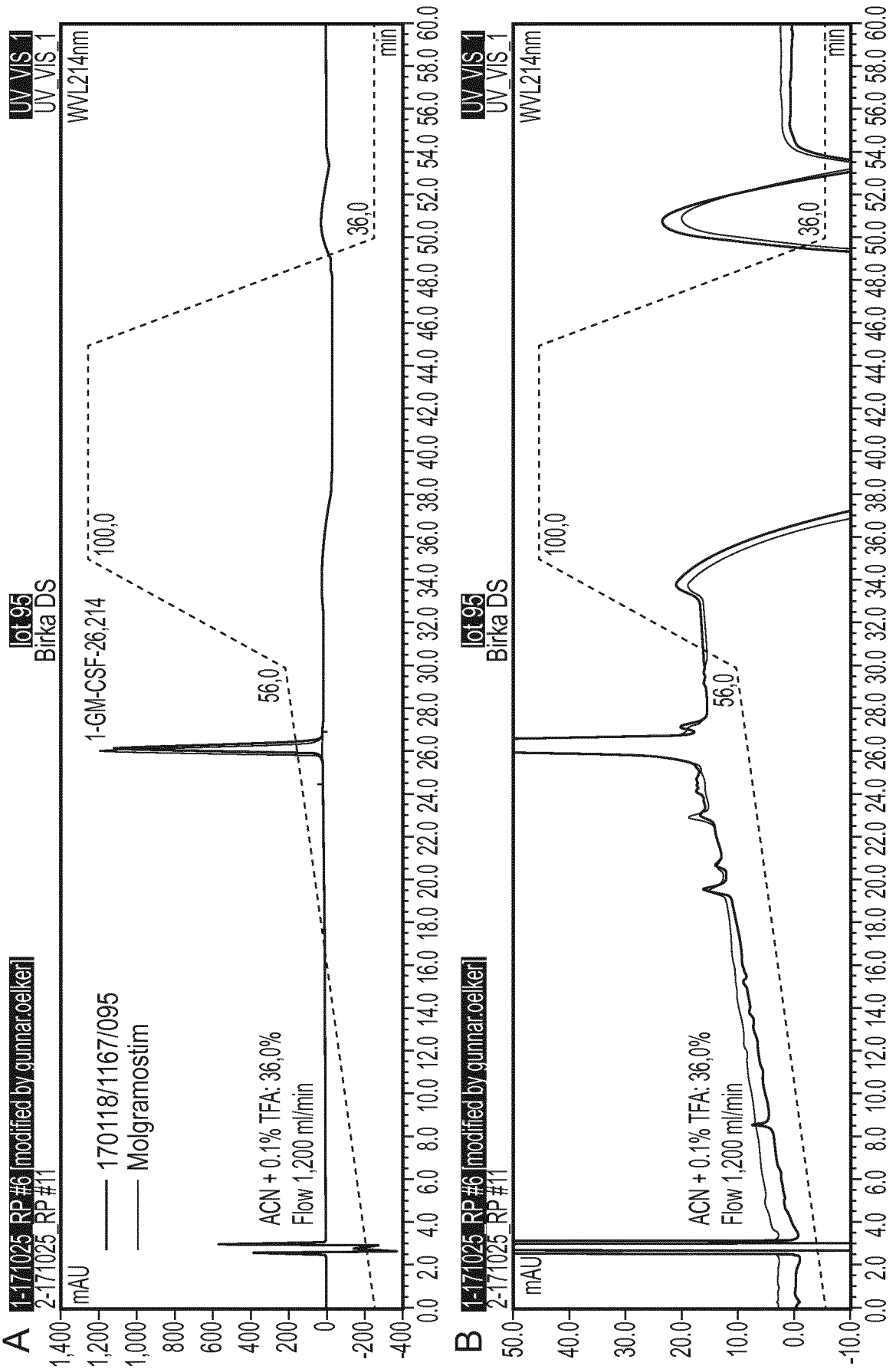
A
1-171025_RP #6 modified by cunnar.oelker
2-171025_RP #11
lot 95
Birka DS
UV VIS 1
UV_VIS_1
WVL214nm
mAU
170118/1167/095
Molgramostim
1-GM-CSF-26,214
100,0
56,0
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
min
B
1-171025_RP #6 modified by cunnar.oelker
2-171025_RP #11
lot 95
Birka DS
UV VIS 1
UV_VIS_1
WVL214nm
mAU
100,0
56,0
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
min

FIG. 30

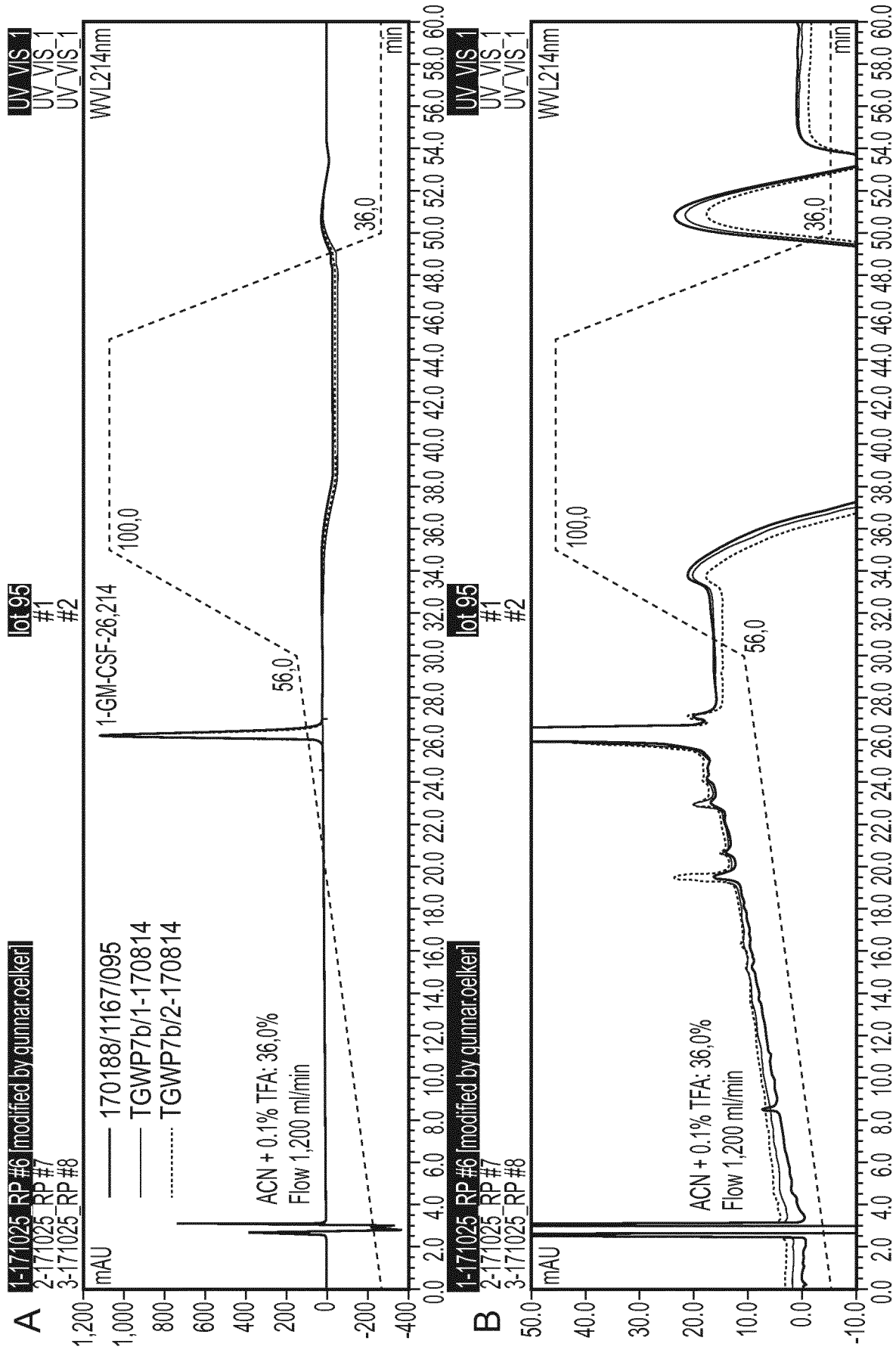
A
B
1-171025_RP #6 [modified by gunnar.oelker]
2-171025_RP #7
3-171025_RP #8
lot 95
#1
#2
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
min
170188/1167/095
TGWP7b/1-170814
TGWP7b/2-170814
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
1-GM-CSF-26,214
100,0
56,0
36,0

FIG. 31

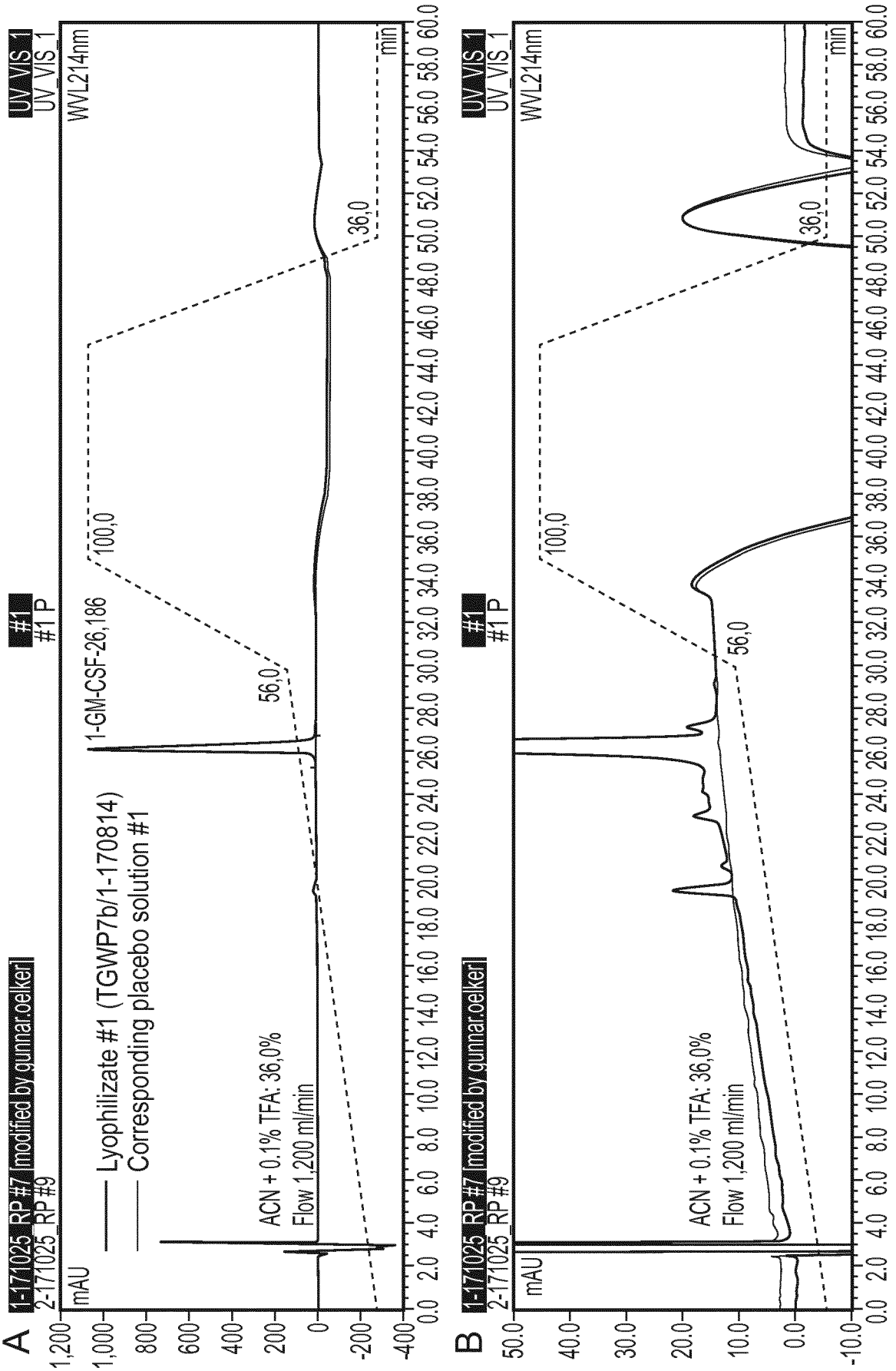
A
1-171025_RP #7 modified by gunnar.oelkerl
2-171025_RP #9
#1
#1 P
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
Lyophilizate #1 (TGWP7b/1-170814)
Corresponding placebo solution #1
1-GM-CSF-26,186
100,0
56,0
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
min
B
1-171025_RP #7 modified by gunnar.oelkerl
2-171025_RP #9
#1
#1 P
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
100,0
56,0
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
min

FIG. 32

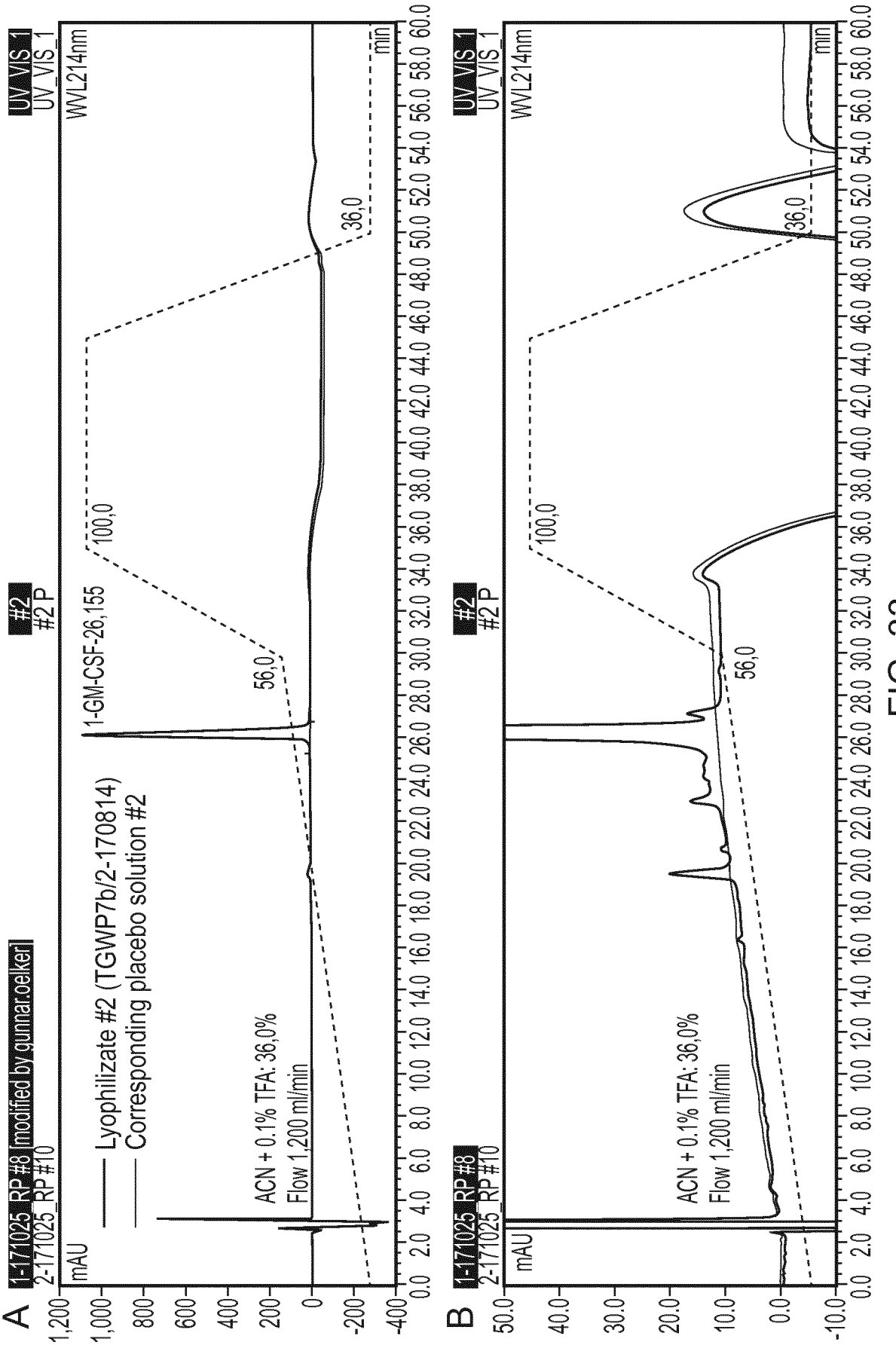
A
1-171025_RP #8 modified by gunnar.oelker
2-171025_RP #10
#2
#2 P
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
min
Lyophilizate #2 (TGWP7b/2-170814)
Corresponding placebo solution #2
1-GM-CSF-26,155
100,0
56,0
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
B
1-171025_RP #8
2-171025_RP #10
#2
#2 P
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
min
100,0
56,0
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min

FIG. 33

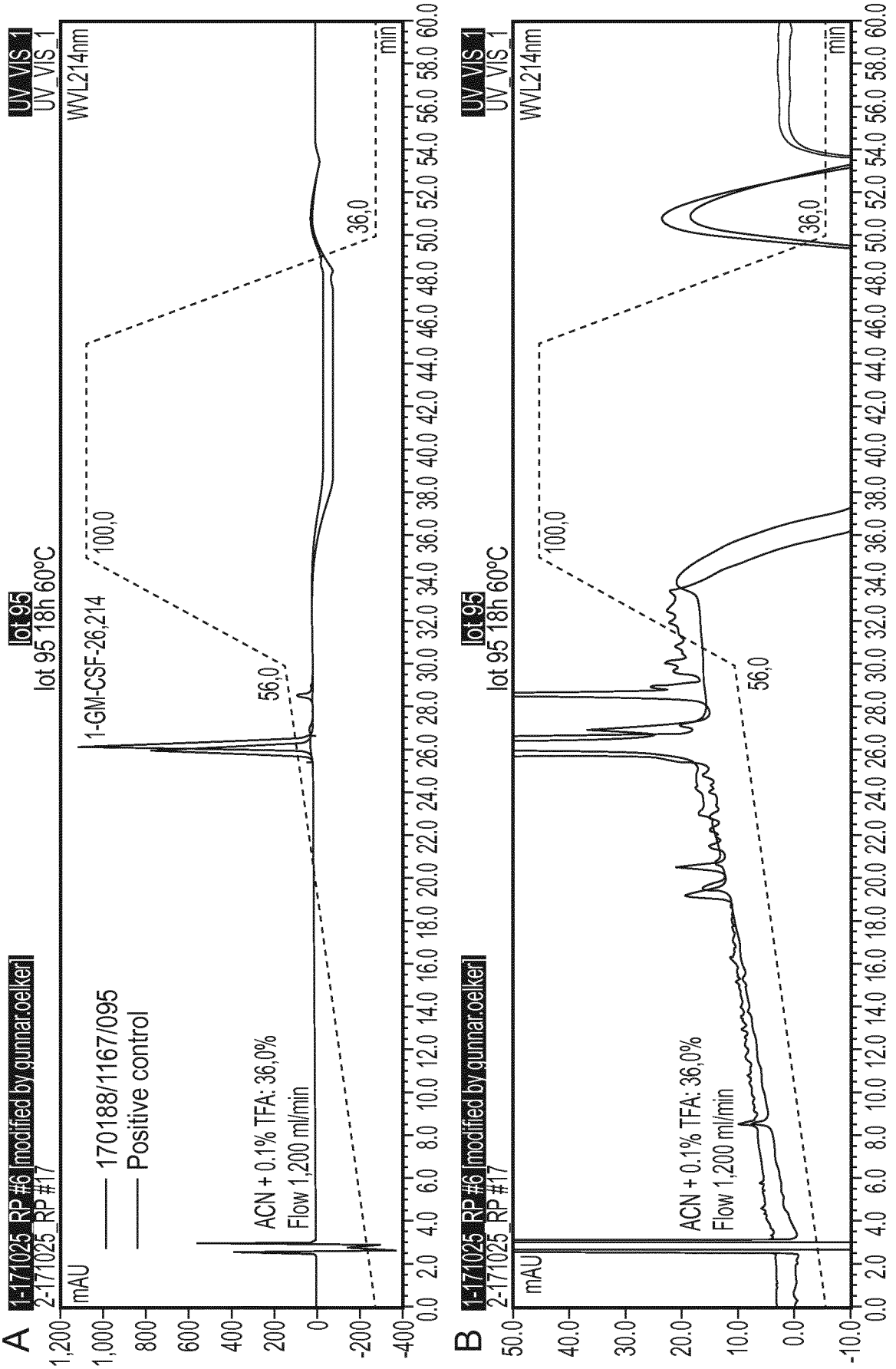
A
1-171025_RP #6 modified by cunnar.oelker
2-171025_RP #17
lot 95
lot 95 18h 60°C
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
min
170188/1167/095
Positive control
1-GM-CSF-26,214
100,0
56,0
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min
B
1-171025_RP #6 modified by cunnar.oelker
2-171025_RP #17
lot 95
lot 95 18h 60°C
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
min
100,0
56,0
36,0
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min

FIG. 34

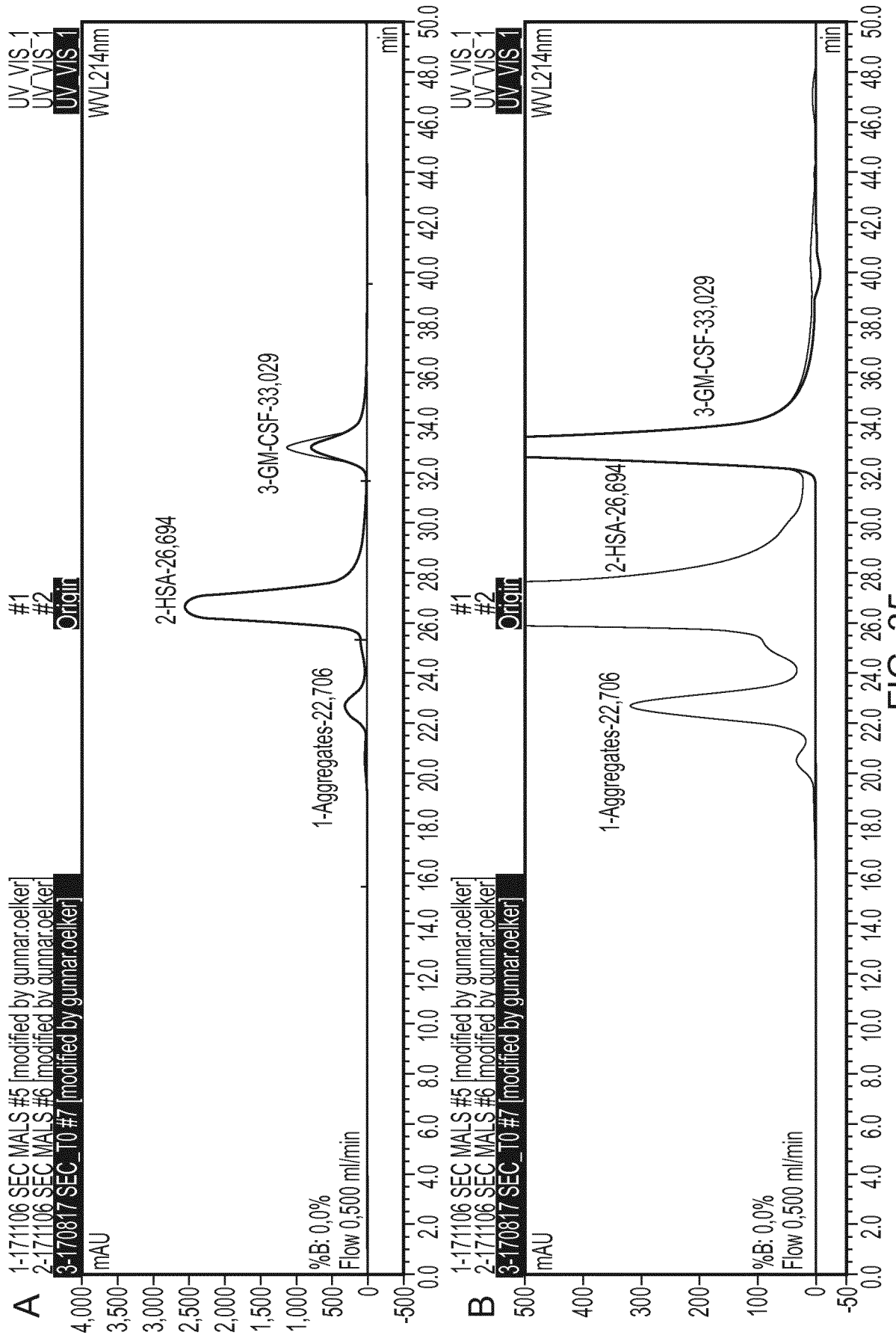
A
1-171106 SEC MALS #5 [modified by gunnar.oelker]
2-171106 SEC MALS #6 [modified by gunnar.oelker]
3-170817 SEC_T0 #7 [modified by gunnar.oelker]
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL:214nm
#1
#2
Origin
mAU
2-HSA-26,694
3-GM-CSF-33,029
1-Aggregates-22,706
%B: 0,0%
Flow 0,500 ml/min
min
B
1-171106 SEC MALS #5 [modified by gunnar.oelker]
2-171106 SEC MALS #6 [modified by gunnar.oelker]
3-170817 SEC_T0 #7 [modified by gunnar.oelker]
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL:214nm
#1
#2
Origin
mAU
2-HSA-26,694
3-GM-CSF-33,029
1-Aggregates-22,706
%B: 0,0%
Flow 0,500 ml/min
min

FIG. 35

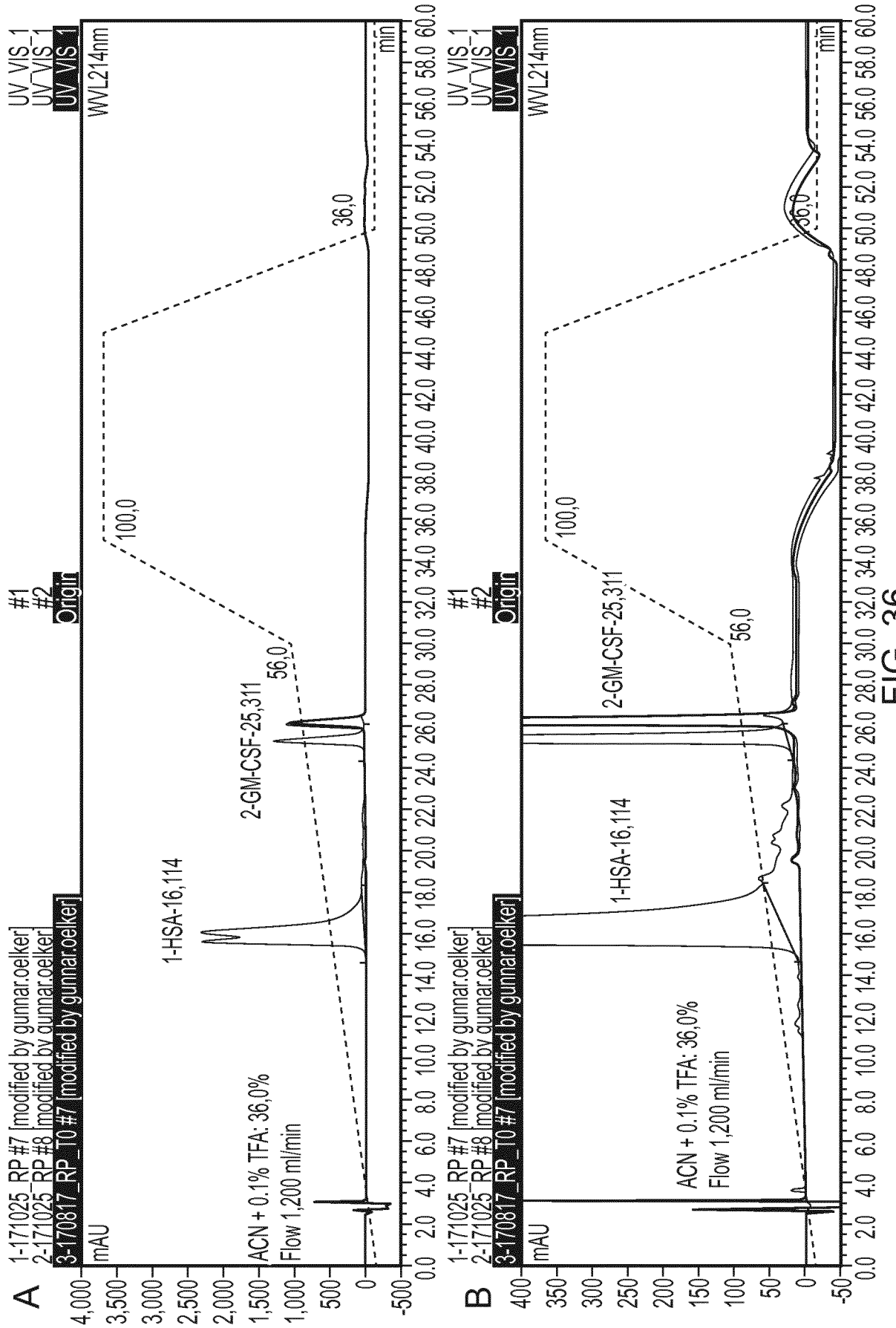
A
B
1-171025_RP #7 [modified by gunnar.oelker]
2-171025_RP #8 [modified by gunnar.oelker]
3-170817_RP_T0 #7 [modified by gunnar.oelker]
UV_VIS_1
UV_VIS_1
UV VIS 1
WVL214nm
mAU
min
#1
#2
Origin
100,0
56,0
36,0
1-HSA-16,114
2-GM-CSF-25,311
ACN + 0.1% TFA: 36,0%
Flow 1,200 ml/min

FIG. 36

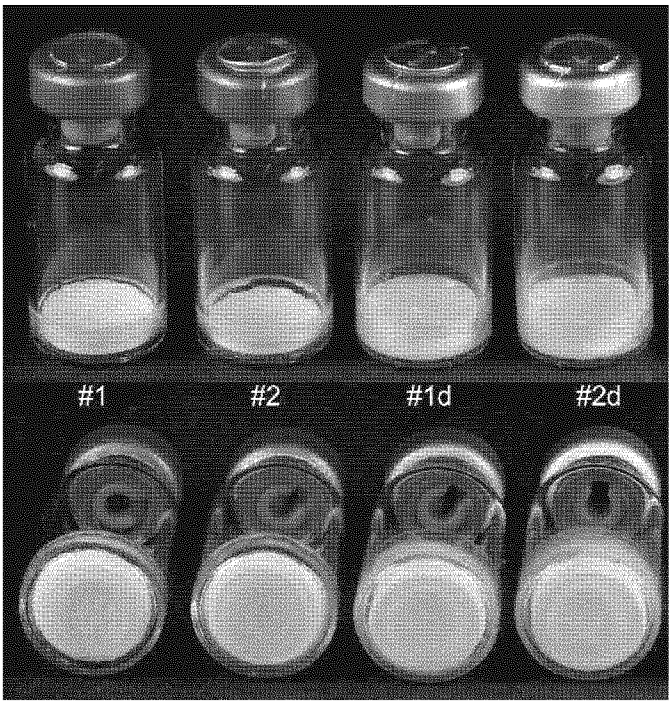

FIG. 37

A B

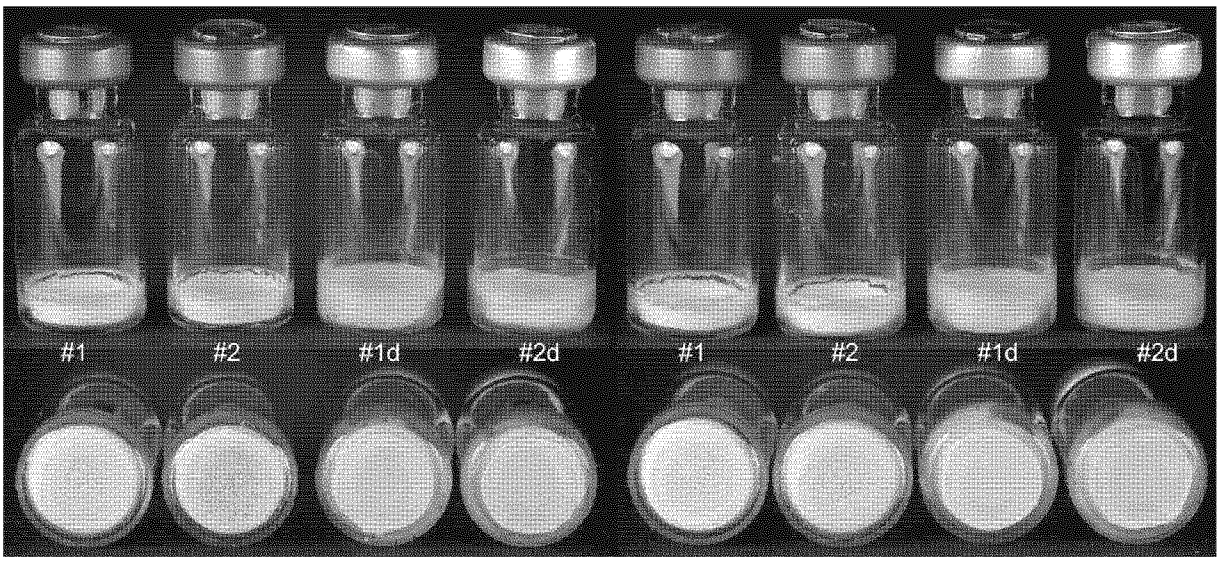

FIG. 38

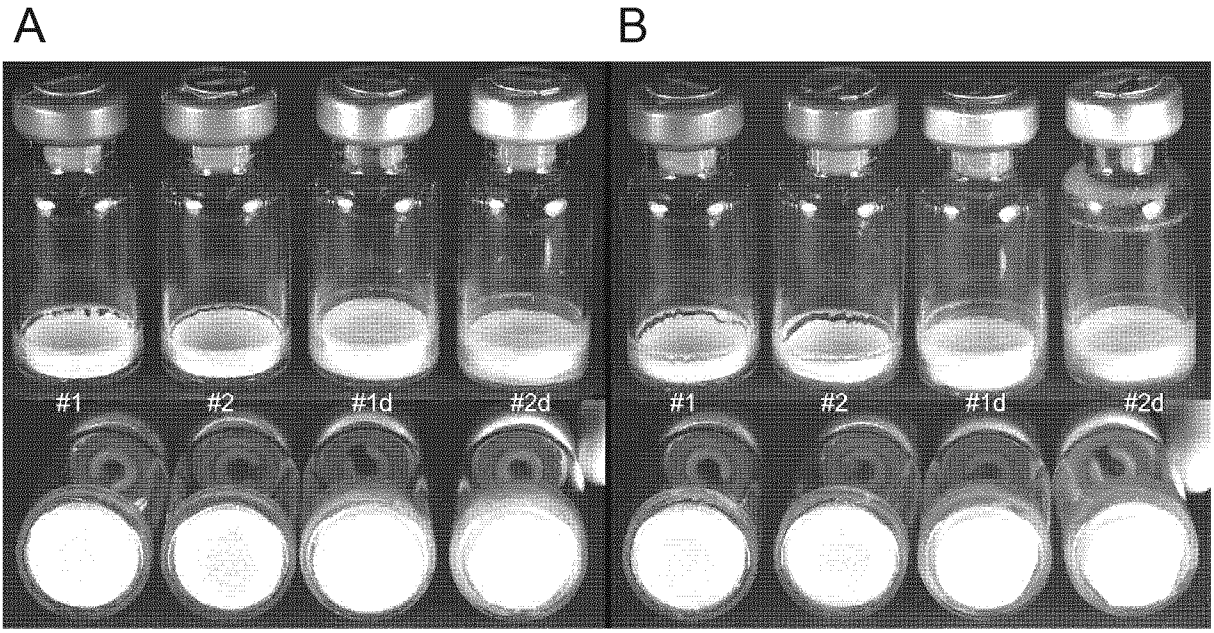

FIG. 39

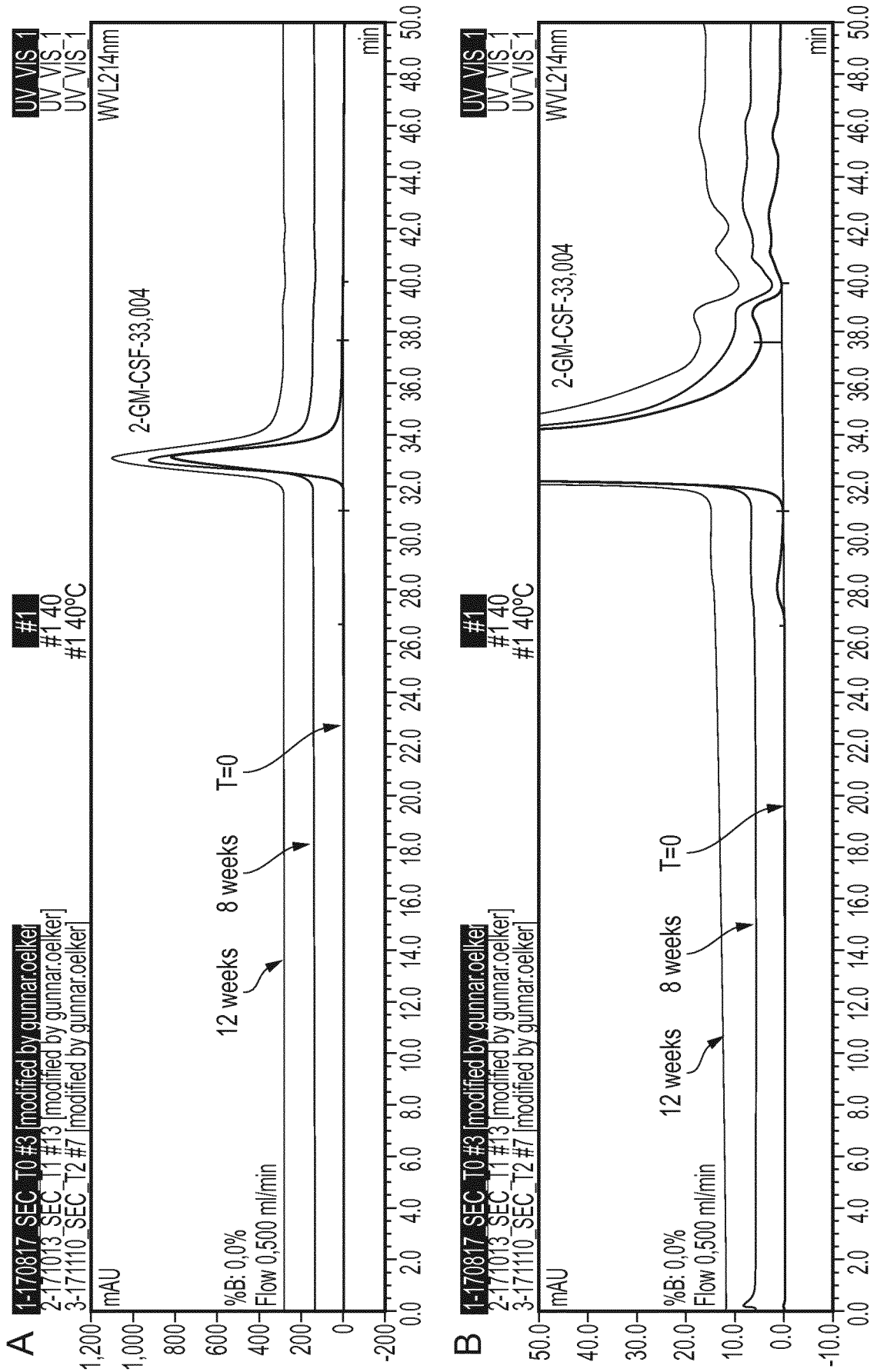
A
1-170817_SEC_T0 #3 [modified by gunnar.oelker]
2-171013_SEC_T1 #13 [modified by gunnar.oelker]
3-171110_SEC_T2 #7 [modified by gunnar.oelker]
#1
#1 40
#1 40°C
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
min
2-GM-CSF-33,004
12 weeks
8 weeks
T=0
%B: 0,0%
Flow 0,500 ml/min
B
1-170817_SEC_T0 #3 [modified by gunnar.oelker]
2-171013_SEC_T1 #13 [modified by gunnar.oelker]
3-171110_SEC_T2 #7 [modified by gunnar.oelker]
#1
#1 40
#1 40°C
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL214nm
mAU
min
2-GM-CSF-33,004
12 weeks
8 weeks
T=0
%B: 0,0%
Flow 0,500 ml/min

FIG. 40

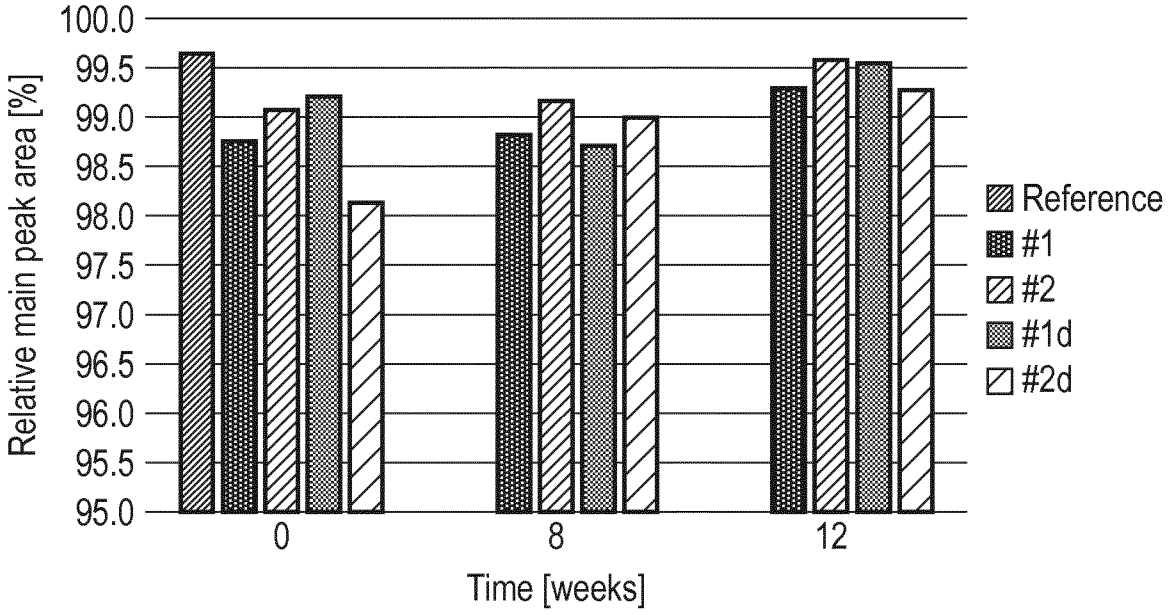
100.0
99.5
99.0
98.5
98.0
97.5
97.0
96.5
96.0
95.5
95.0
Relative main peak area [%]
0
8
12
Time [weeks]
Reference
#1
#2
#1d
#2d

FIG. 41

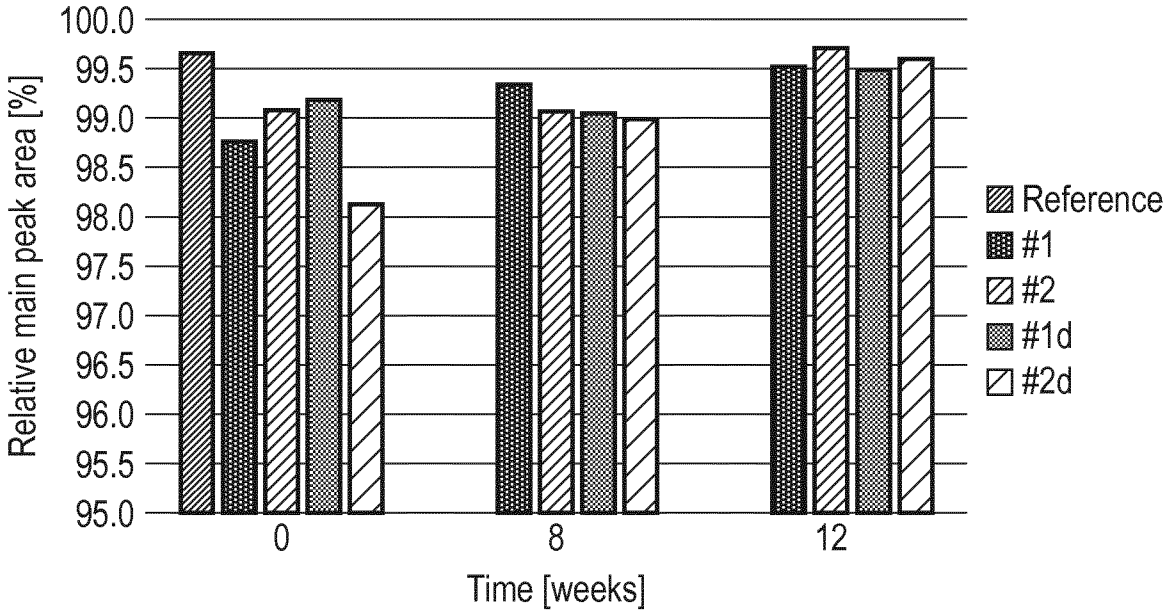
100.0
99.5
99.0
98.5
98.0
97.5
97.0
96.5
96.0
95.5
95.0
Relative main peak area [%]
0
8
12
Time [weeks]
Reference
#1
#2
#1d
#2d

FIG. 42

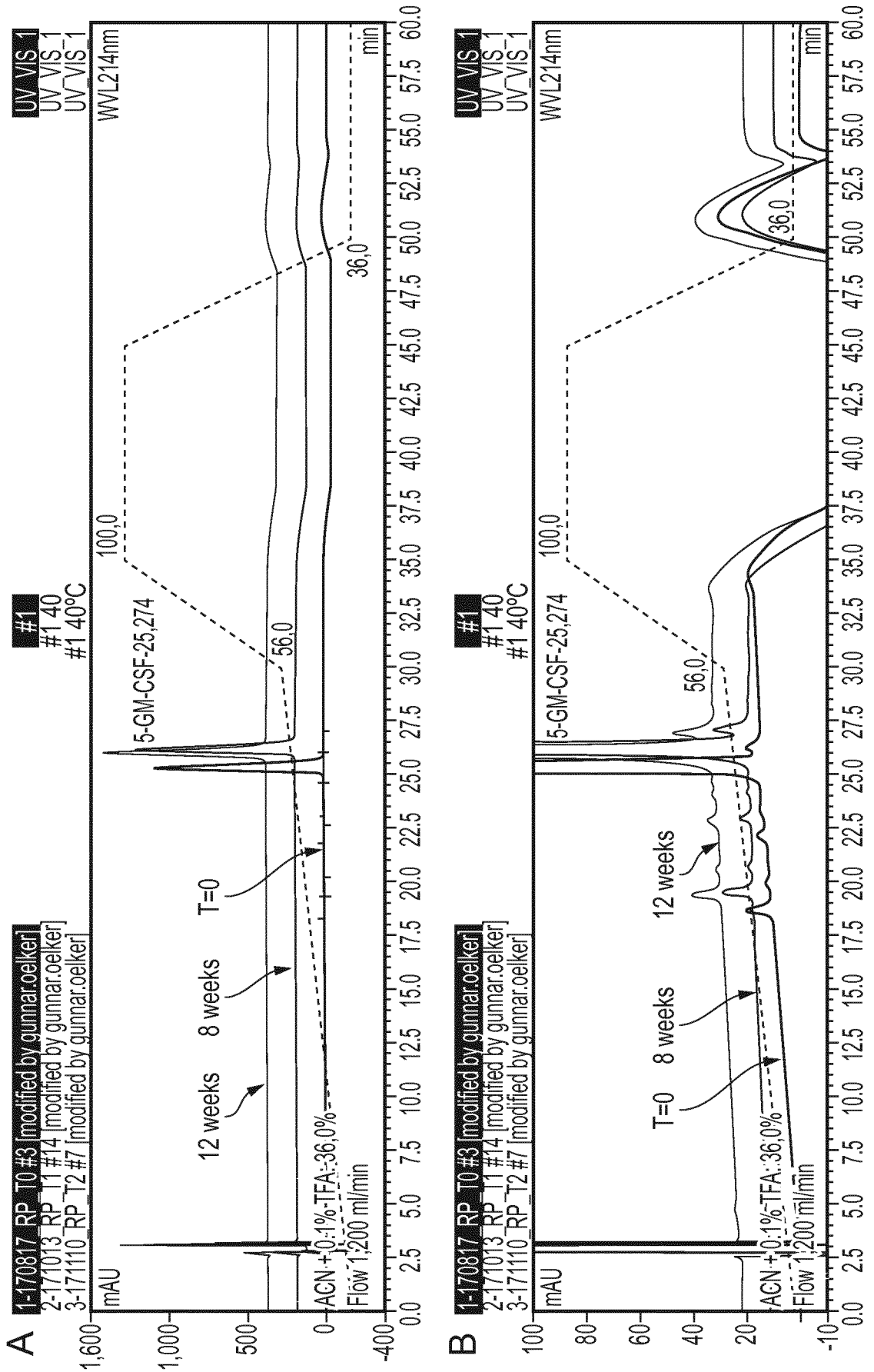
A
B
1-170817_RP_T0 #3 [modified by gunnar.oelker]
2-171013_RP_T1 #14 [modified by gunnar.oelker]
3-171110_RP_T2 #7 [modified by gunnar.oelker]
UV_VIS_1
UV_VIS_1
UV_VIS_1
#1
#1 40
#1 40°C
WVL214nm
mAU
min
5-GM-CSF-25,274
12 weeks
8 weeks
T=0
56,0
36,0
100,0
ACN + 0.1%-TFA: 36,0%
Flow 1,200 ml/min

FIG. 43

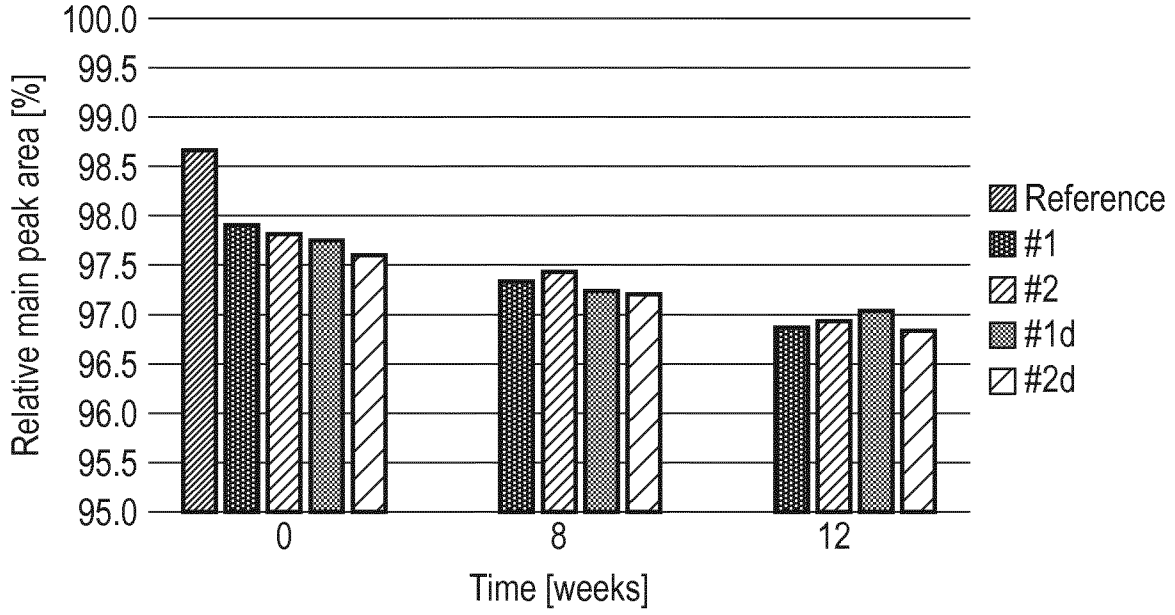
100.0
99.5
99.0
98.5
98.0
97.5
97.0
96.5
96.0
95.5
95.0
Relative main peak area [%]
0
8
12
Time [weeks]
Reference
#1
#2
#1d
#2d

FIG. 44

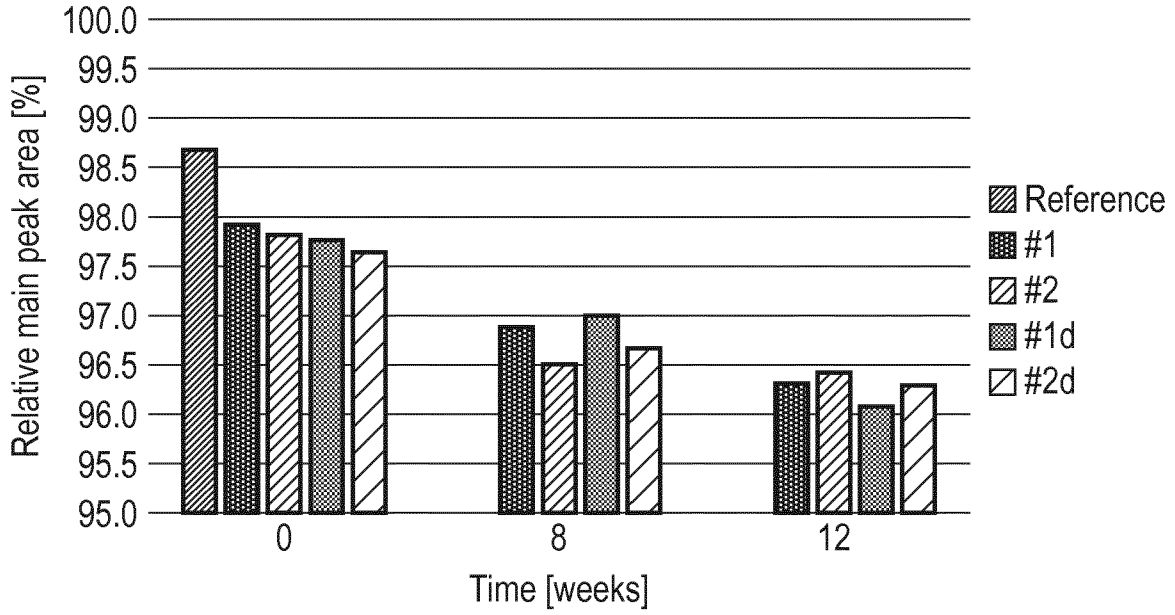
100.0
99.5
99.0
98.5
98.0
97.5
97.0
96.5
96.0
95.5
95.0
Relative main peak area [%]
0
8
12
Time [weeks]
Reference
#1
#2
#1d
#2d

FIG. 45

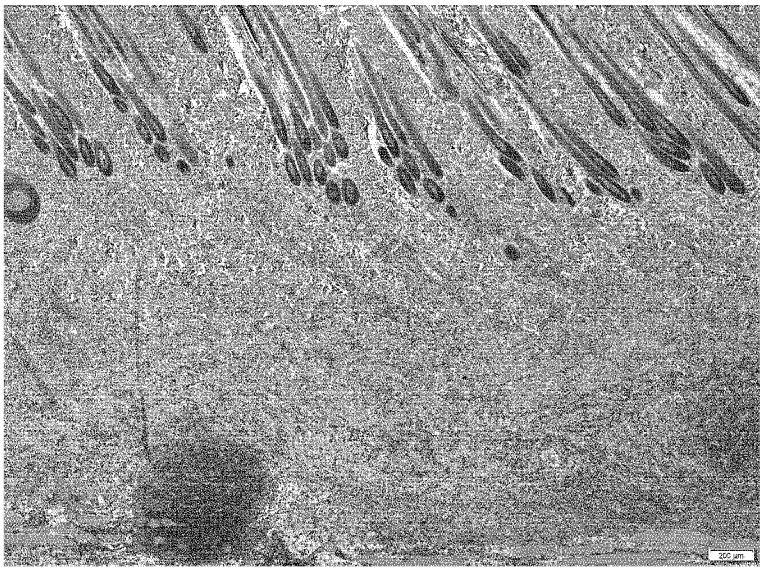

FIG. 46

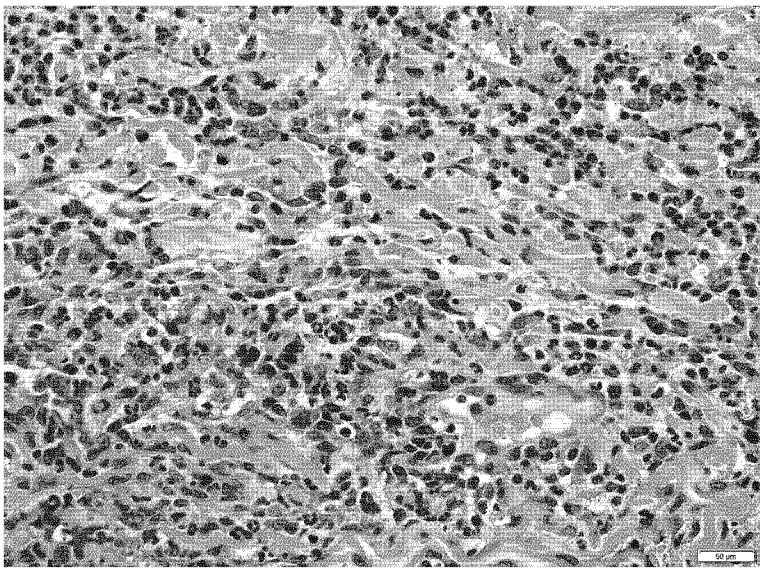

FIG. 47

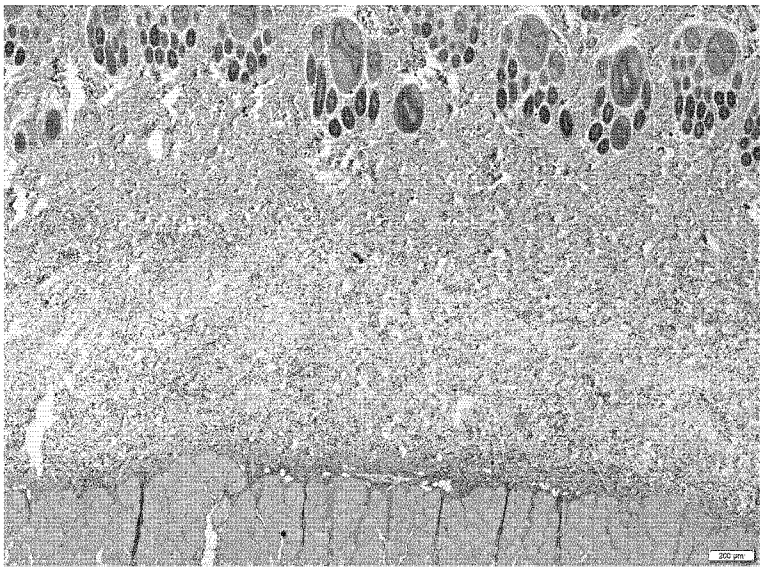

FIG. 48

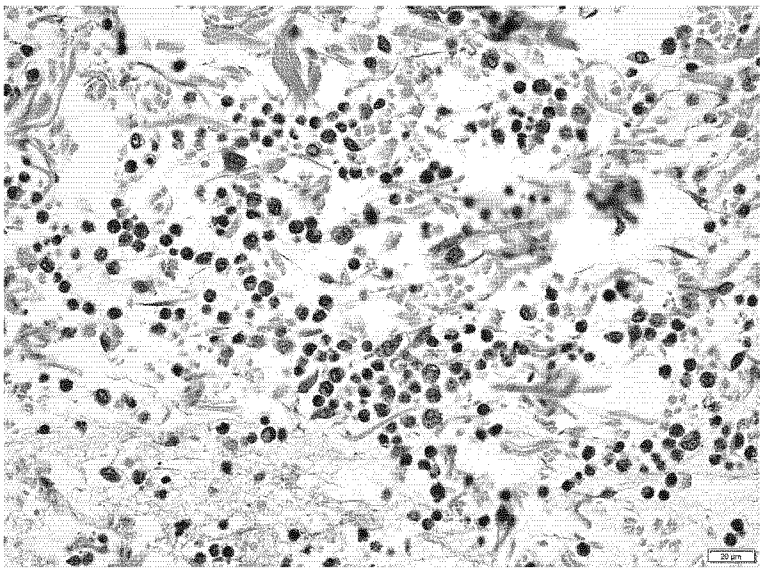

FIG. 49

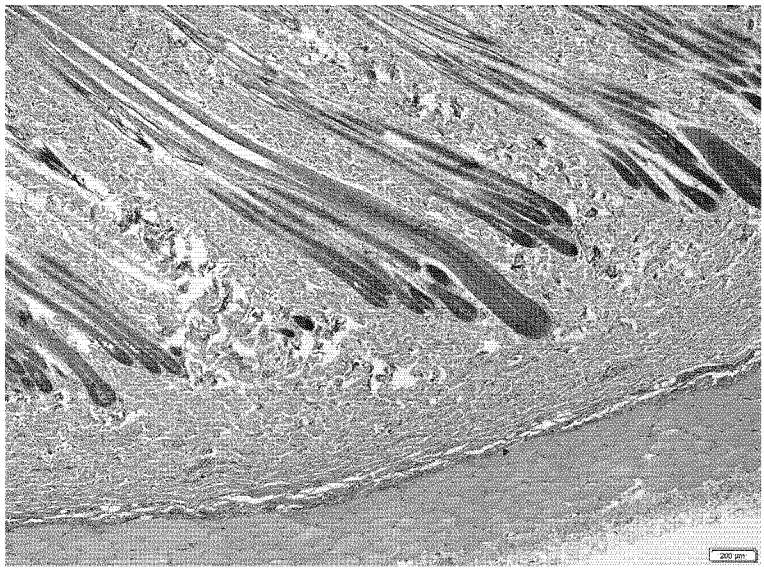

FIG. 50

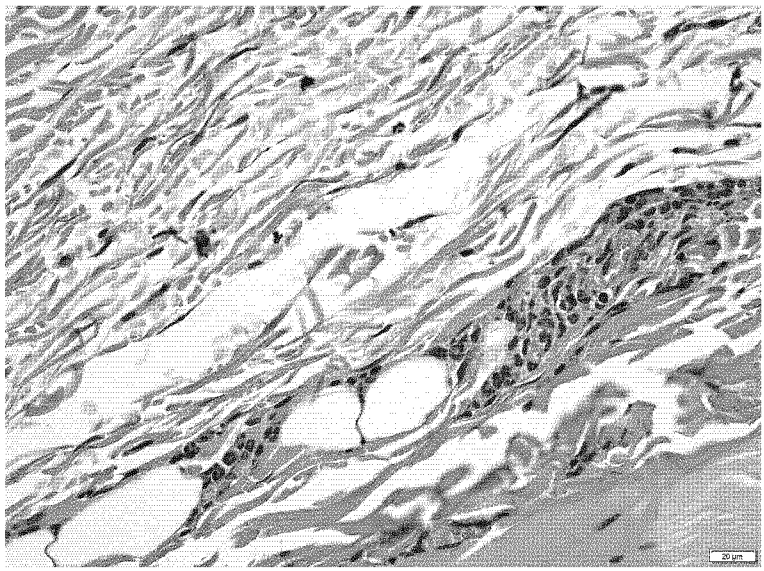

FIG. 51

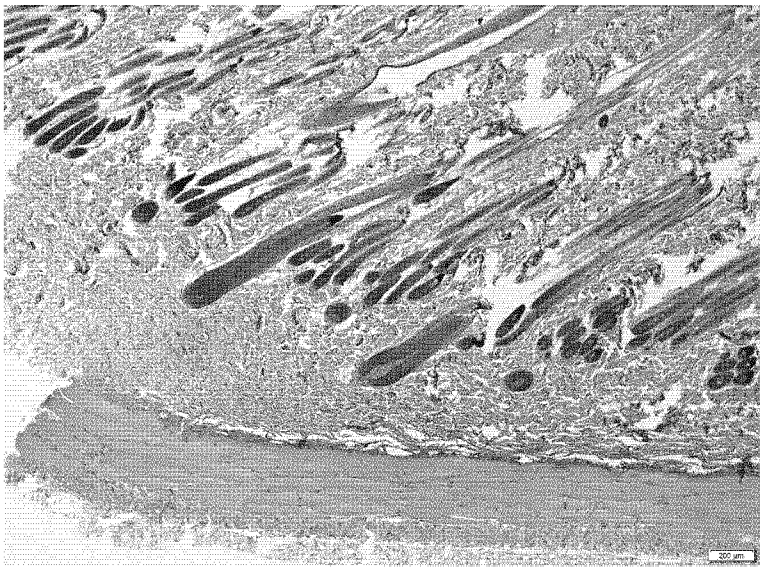

FIG. 52

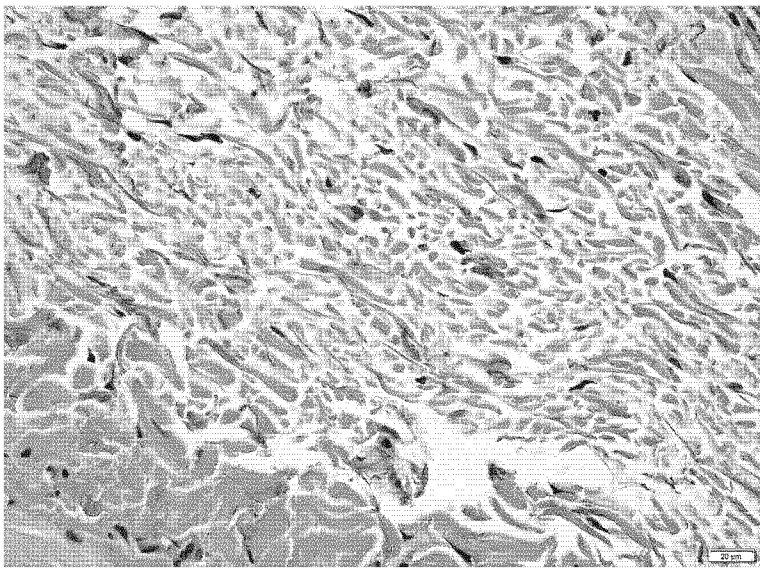

FIG. 53

FIG. 54

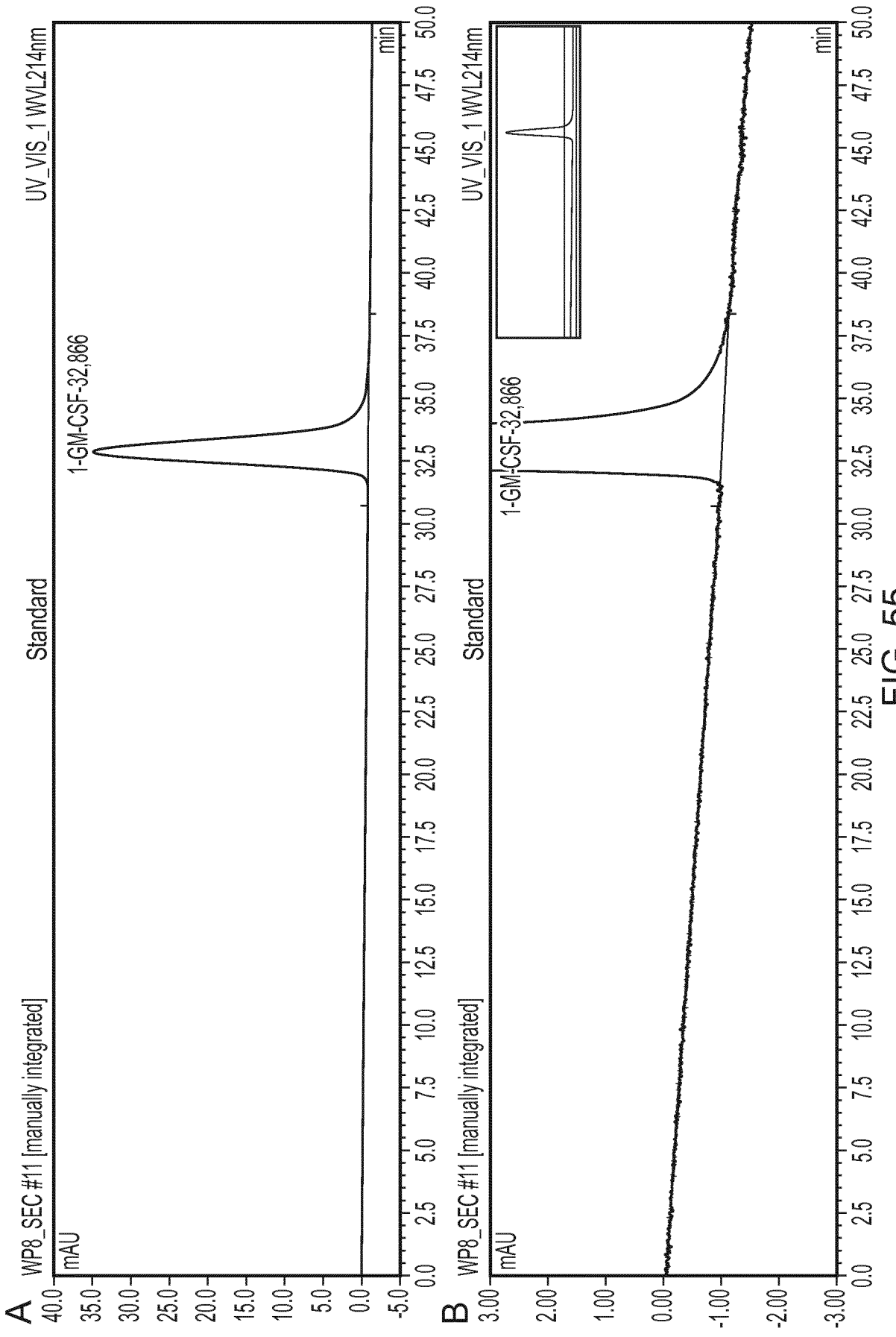
A
WP8_SEC #11 [manually integrated]
UV_VIS_1 WVL214nm
Standard
mAU
1-GM-CSF-32,866
min
B
WP8_SEC #11 [manually integrated]
UV_VIS_1 WVL214nm
Standard
mAU
1-GM-CSF-32,866
min

FIG. 55

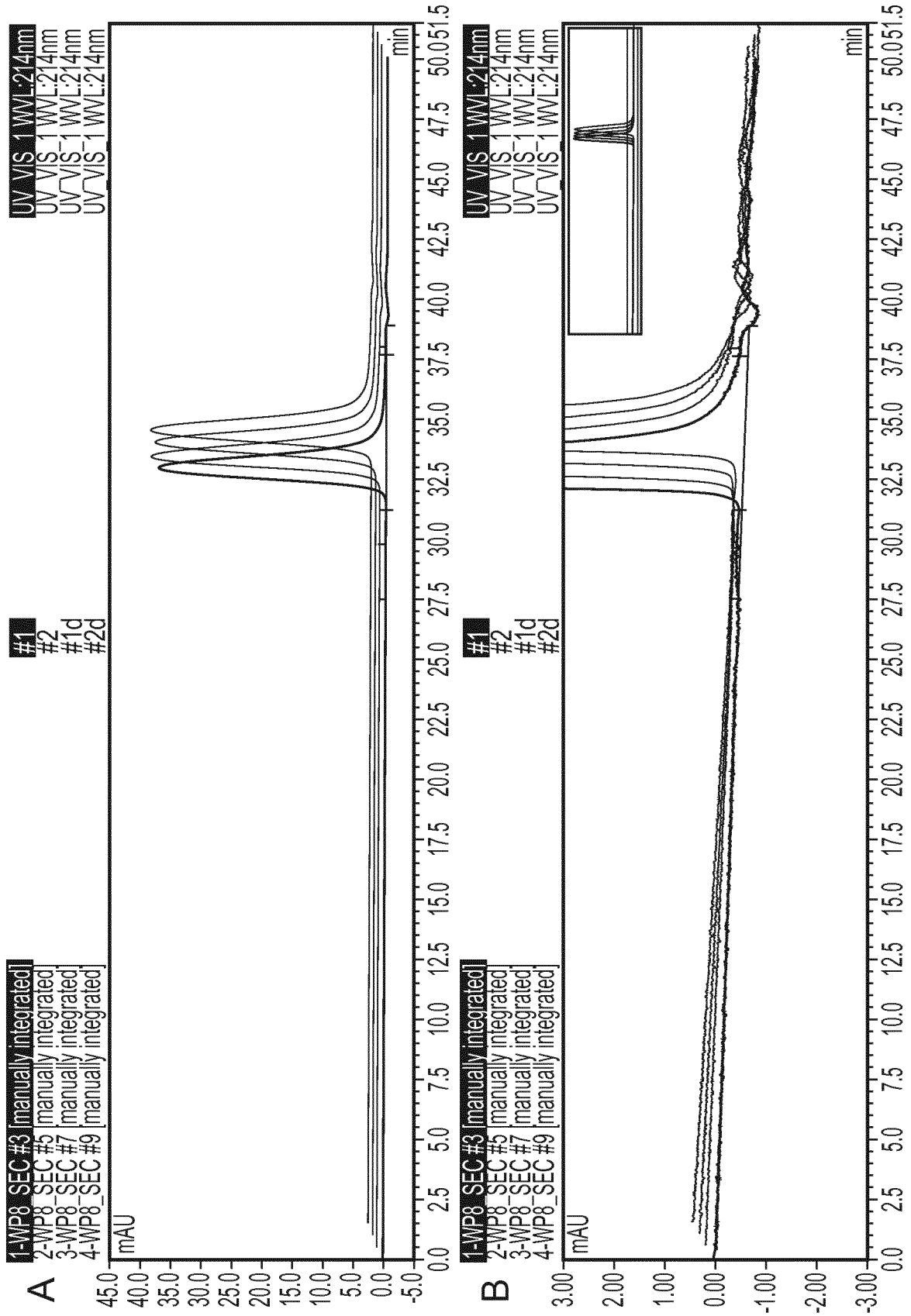
A
B
1-WP8_SEC #3 [manually integrated]
2-WP8_SEC #5 [manually integrated]
3-WP8_SEC #7 [manually integrated]
4-WP8_SEC #9 [manually integrated]
UV_VIS 1 WVL:214nm
UV_VIS_1 WVL:214nm
UV_VIS_1 WVL:214nm
UV_VIS_1 WVL:214nm
#1
#2
#1d
#2d
mAU
min

FIG. 56

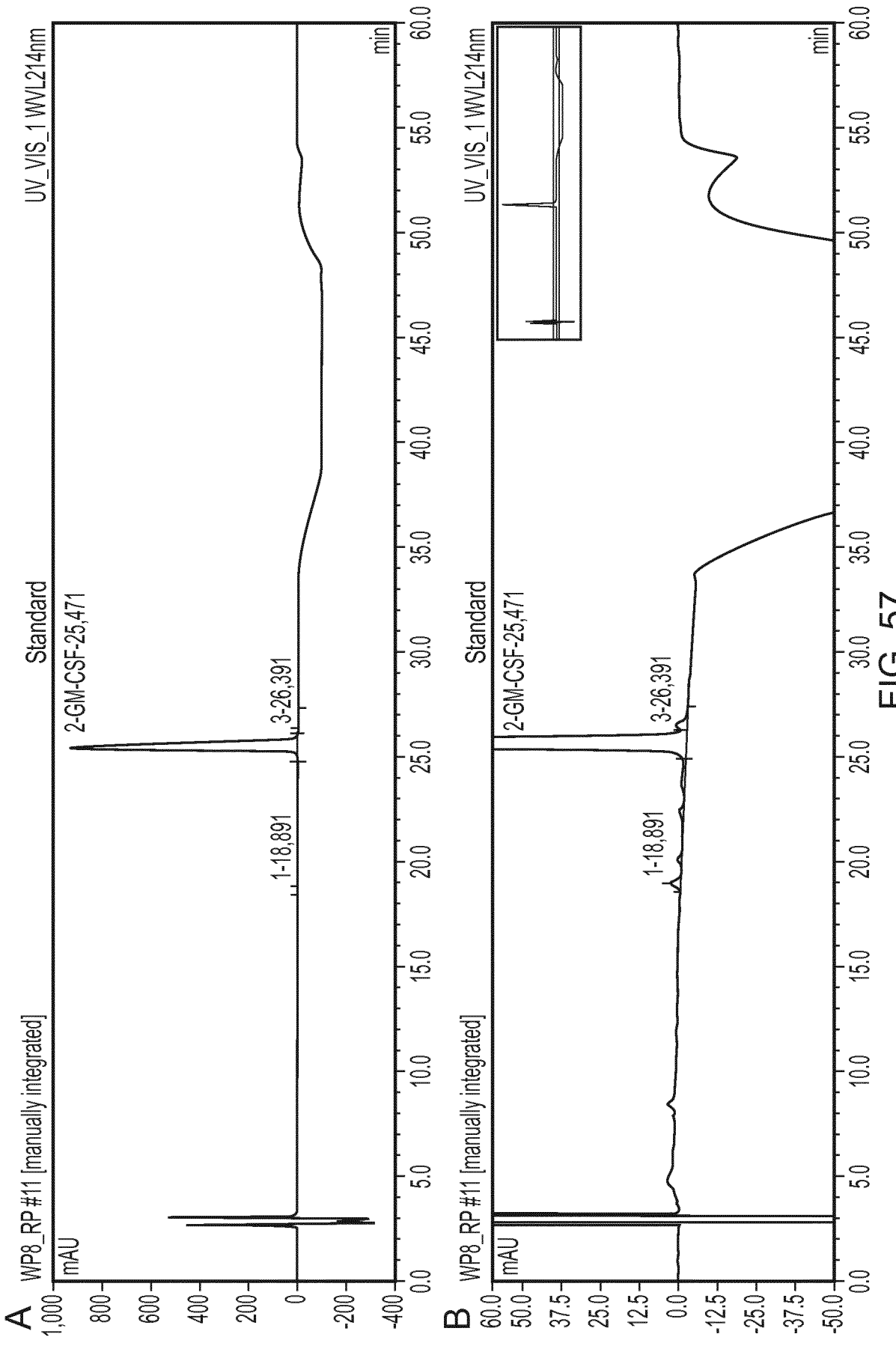
A
WP8_RP #11 [manually integrated]
UV_VIS_1 WVL214nm
Standard
mAU
min
2-GM-CSF-25,471
3-26,391
1-18,891
B
WP8_RP #11 [manually integrated]
UV_VIS_1 WVL214nm
Standard
mAU
min
2-GM-CSF-25,471
3-26,391
1-18,891

FIG. 57

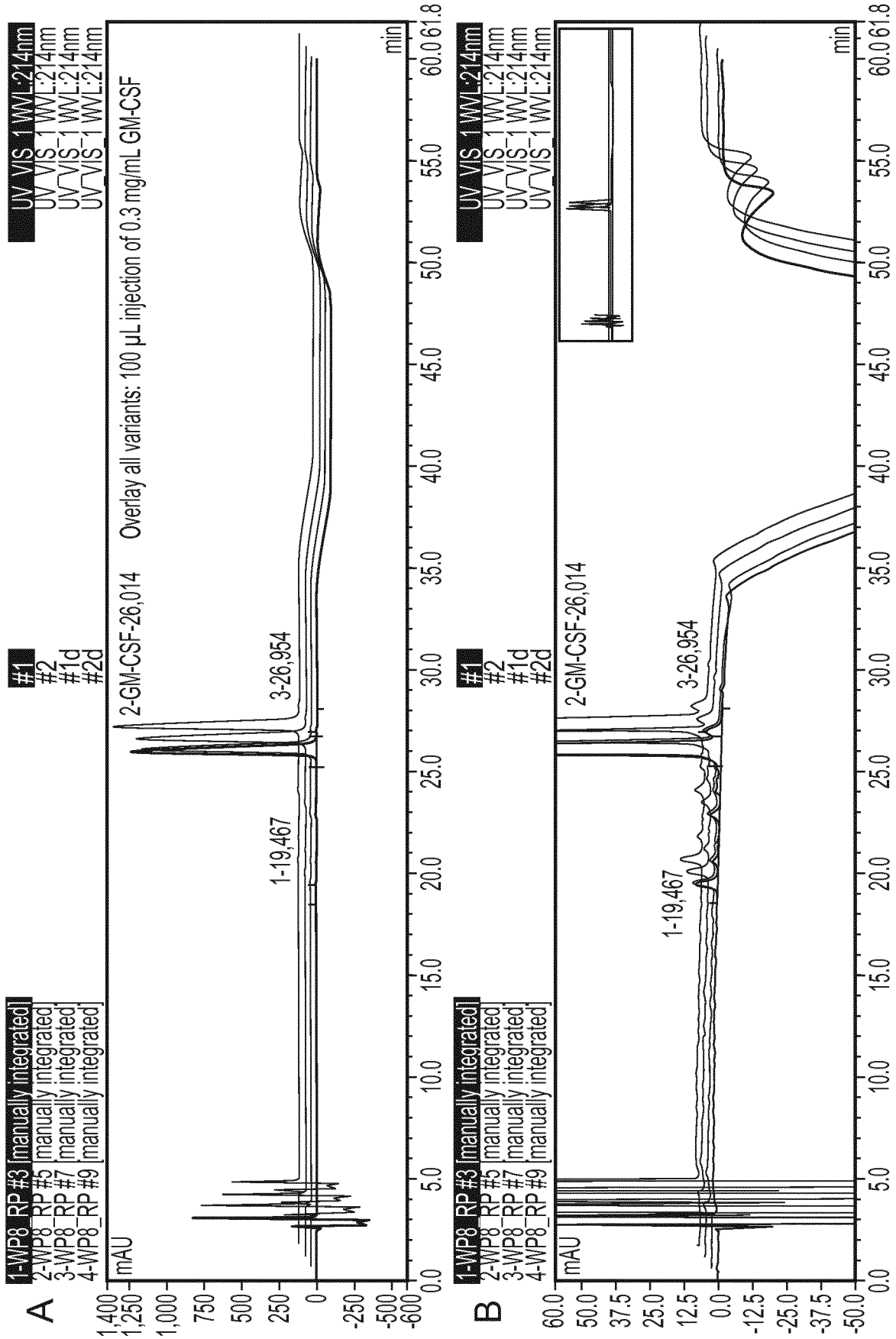
A
B
1-WP8_RP #3 [manually integrated]
2-WP8_RP #5 [manually integrated]
3-WP8_RP #7 [manually integrated]
4-WP8_RP #9 [manually integrated]
UV_VIS_1 WVL:214nm
UV_VIS_1 WVL:214nm
UV_VIS_1 WVL:214nm
UV_VIS_1 WVL:214nm
#1
#2
#1d
#2d
2-GM-CSF-26,014
Overlay all variants: 100 µL injection of 0.3 mg/mL GM-CSF
3-26,954
1-19,467
mAU
min

FIG. 58

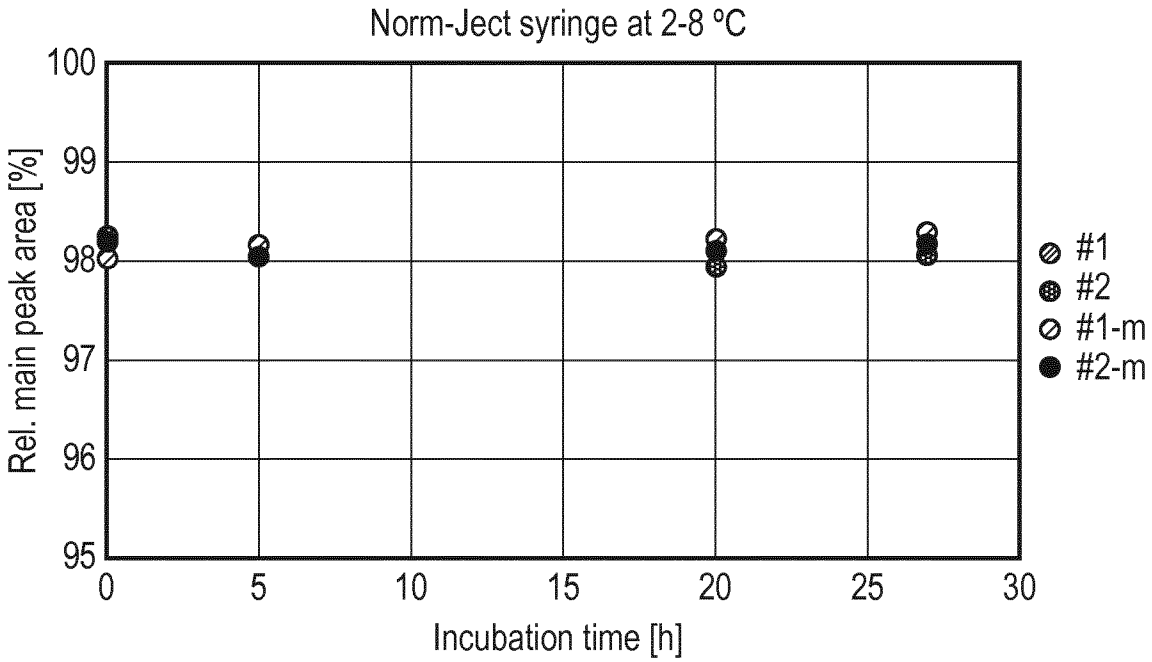
Norm-Ject syringe at 2-8 °C
Rel. main peak area [%]
100
99
98
97
96
95
0
5
10
15
20
25
30
Incubation time [h]
#1
#2
#1-m
#2-m

FIG. 59

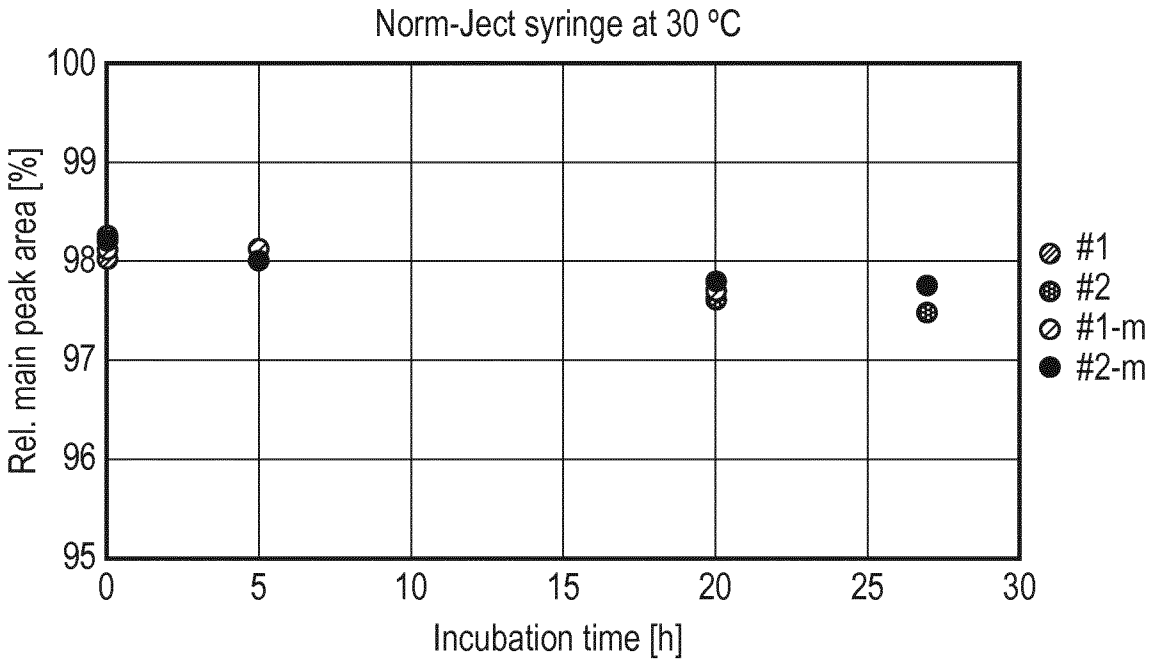
Norm-Ject syringe at 30 °C
Rel. main peak area [%]
100
99
98
97
96
95
0
5
10
15
20
25
30
Incubation time [h]
#1
#2
#1-m
#2-m

FIG. 60

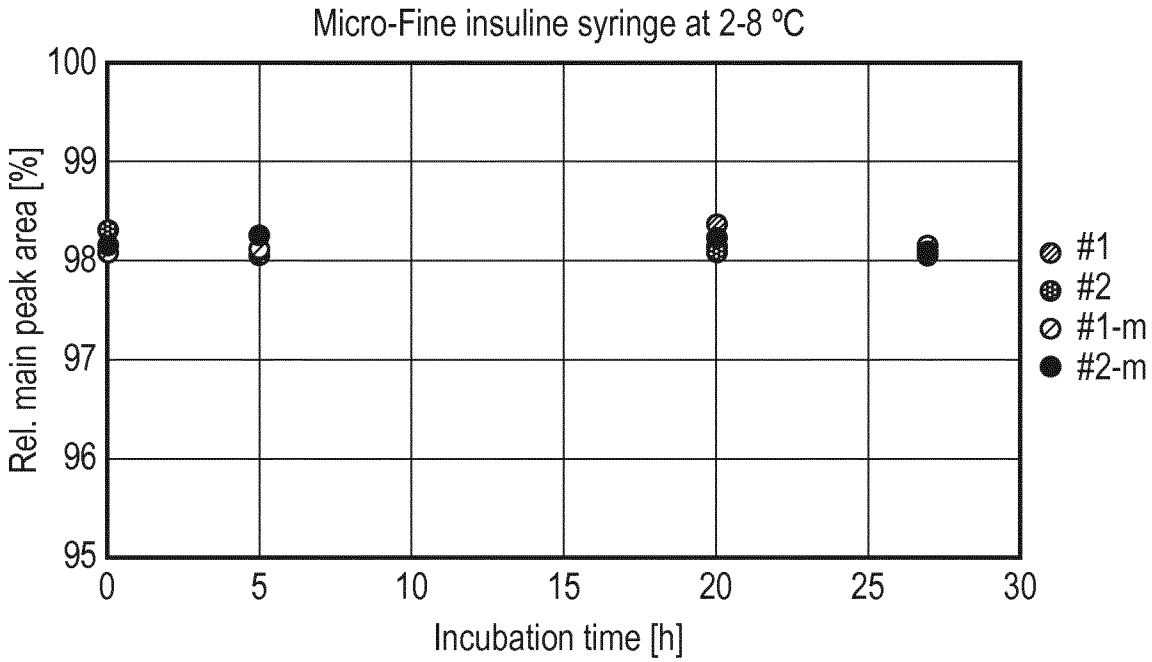
Micro-Fine insuline syringe at 2-8 °C
Rel. main peak area [%]
100
99
98
97
96
95
0
5
10
15
20
25
30
Incubation time [h]
#1
#2
#1-m
#2-m

FIG. 61

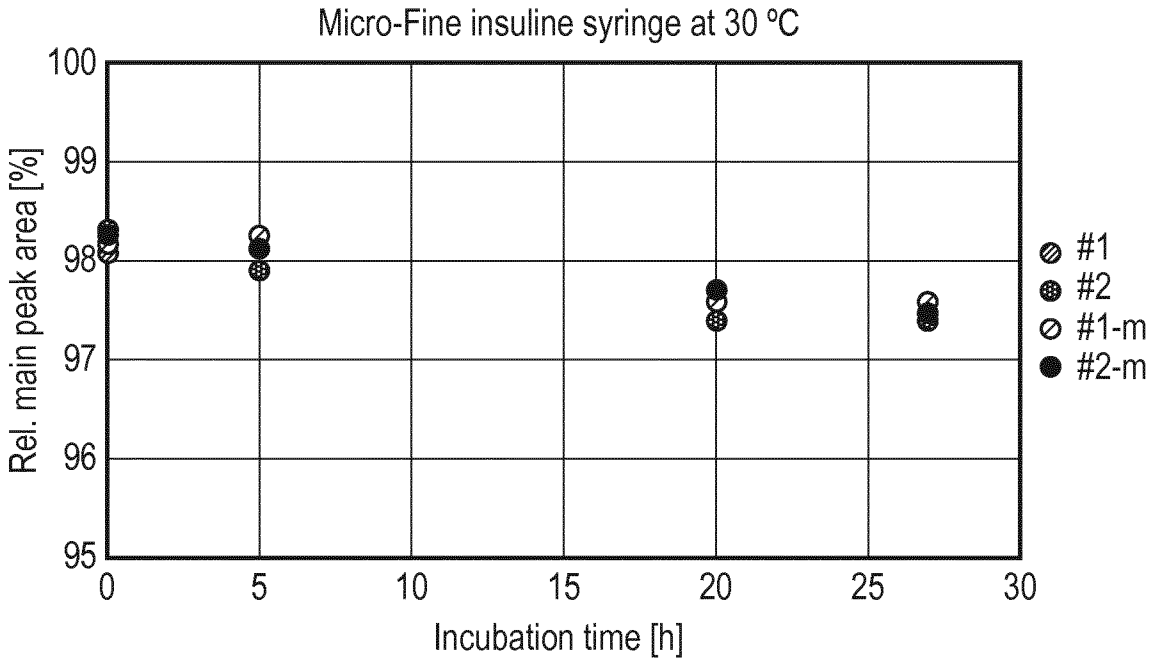
Micro-Fine insuline syringe at 30 °C
Rel. main peak area [%]
100
99
98
97
96
95
0
5
10
15
20
25
30
Incubation time [h]
#1
#2
#1-m
#2-m

FIG. 62

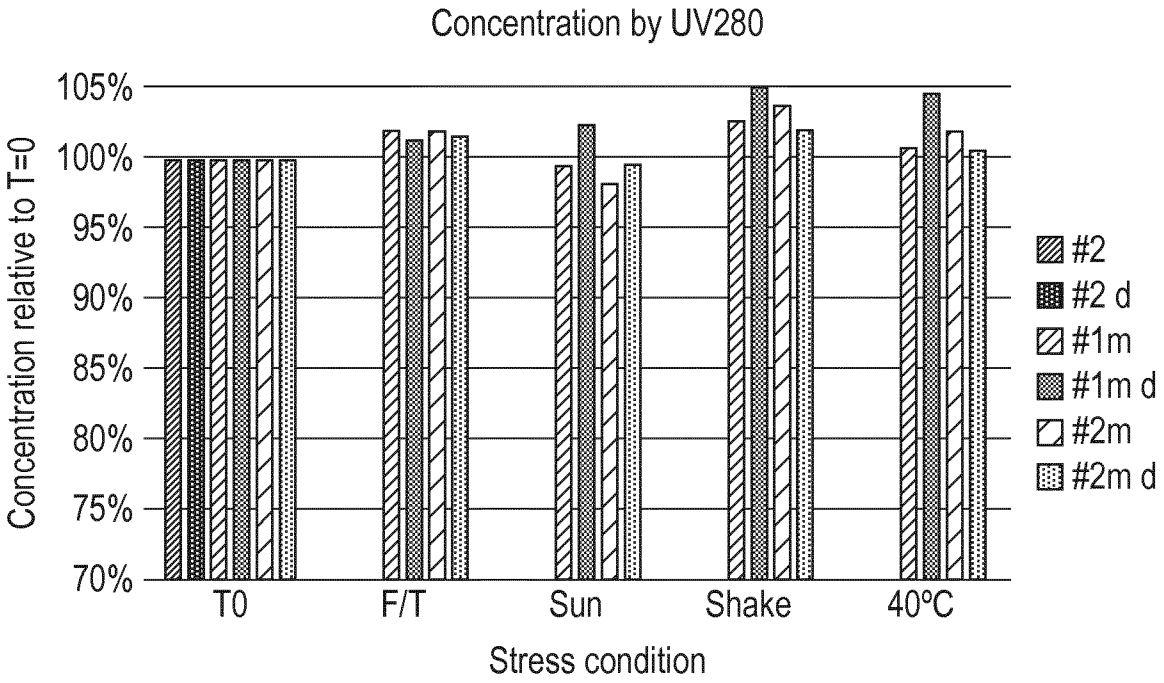
Concentration by UV280
Concentration relative to T=0
105%
100%
95%
90%
85%
80%
75%
70%
T0
F/T
Sun
Shake
40°C
Stress condition
#2
#2 d
#1m
#1m d
#2m
#2m d

FIG. 63

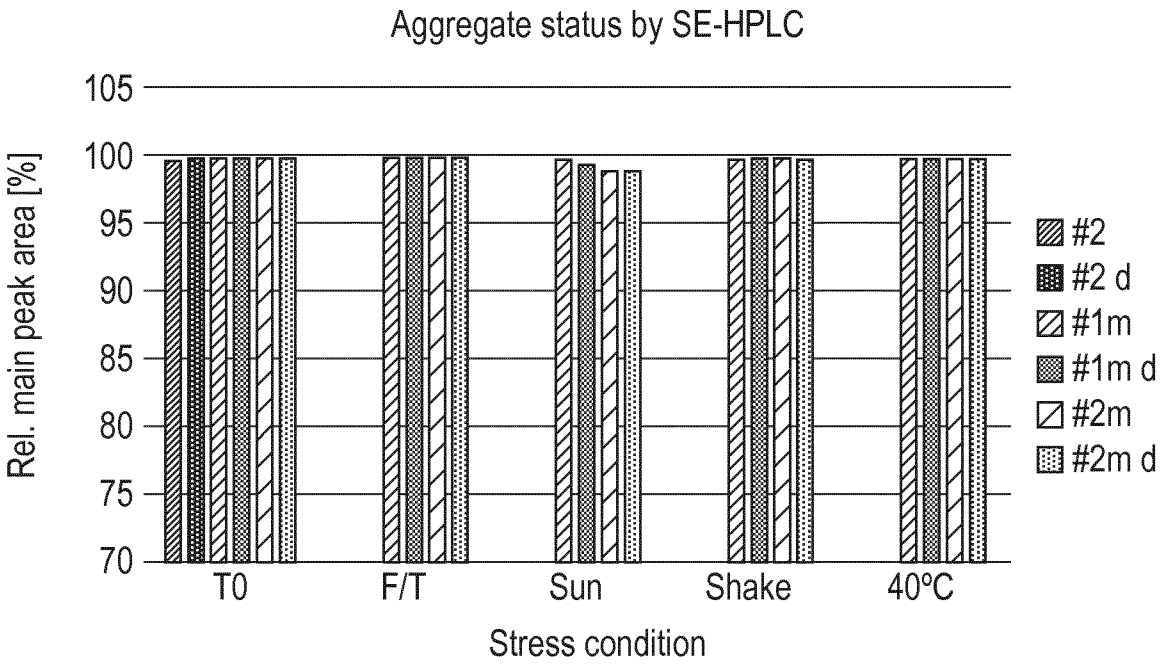
Aggregate status by SE-HPLC
Rel. main peak area [%]
105
100
95
90
85
80
75
70
T0
F/T
Sun
Shake
40°C
Stress condition
#2
#2 d
#1m
#1m d
#2m
#2m d

FIG. 64

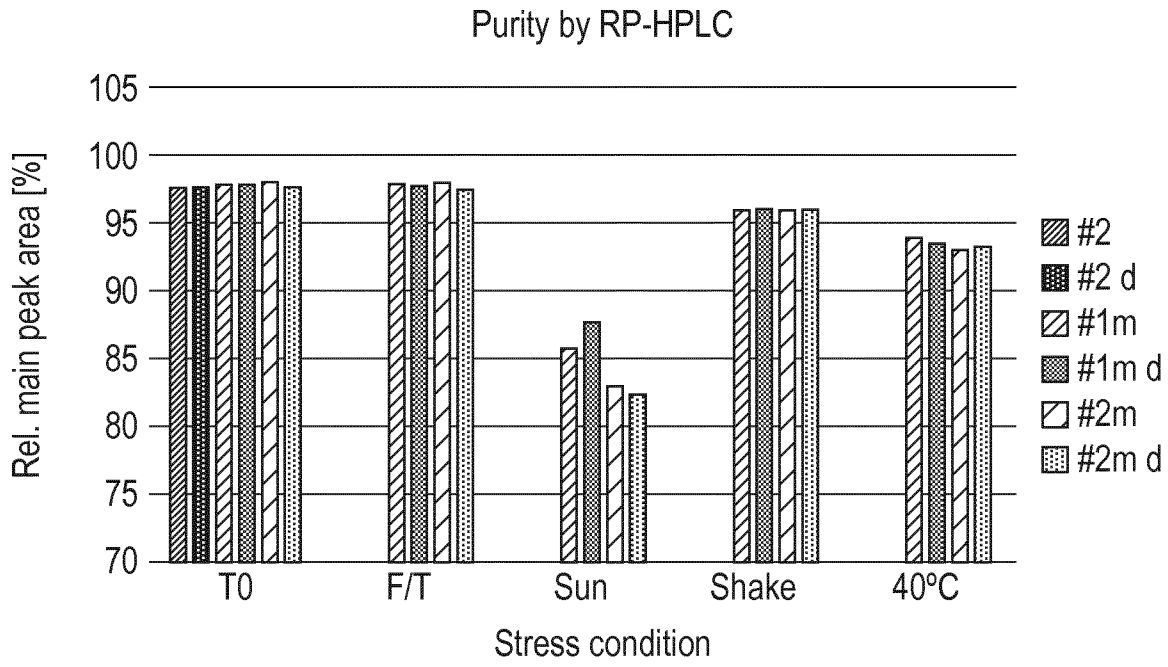
Purity by RP-HPLC
Rel. main peak area [%]
105
100
95
90
85
80
75
70
T0
F/T
Sun
Shake
40°C
Stress condition
#2
#2 d
#1m
#1m d
#2m
#2m d

FIG. 65

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/067428

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/08 A61K9/00 A61K47/10 A61K47/26 A61K38/19
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KR 2013 0020760 A (BCWORLD PHARM CO LTD [KR]) 28 February 2013 (2013-02-28) formulation 1-8, 1-9; paragraph [0075]	1-6, 11-13,20
Y	WO 93/05799 A1 (ERBA CARLO SPA [IT]) 1 April 1993 (1993-04-01) abstract; claim 12; examples 1-3; tables 1-4; sequence 1=3	1-21
Y	WO 02/069999 A1 (IMMUNEX CORP [US]; PETTIT DEAN K [US]; JOCHHEIM CLAUDIA M [US]) 12 September 2002 (2002-09-12) abstract; claims 1-9; examples 1-3	1-21

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 2019	16/10/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Madalinska, K

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 1 598 074 A1 (CHUGAI PHARMACEUTICAL CO LTD [JP]) 23 November 2005 (2005-11-23) abstract; claims 1-11; figures 1-3; examples 1-6 paragraph [10+13]	1-21
Y	WO 2008/122415 A1 (SANDOZ AG [CH]; FUERTINGER SABINE [AT]; MATOUS HEINRICH [AT]) 16 October 2008 (2008-10-16) abstract; claims 1-17; example 1	1-21
A	WO 2005/013933 A1 (UNIV LIEGE [BE]; HUBERT PASCALE [BE]; EVRARD BRIGITTE [BE]; DELATTRE L) 17 February 2005 (2005-02-17) figure 1; examples 9, 10	1-21
A	WO 97/13502 A2 (IMMUNEX CORP [US]; AMERICAN CYANAMID CO [US]) 17 April 1997 (1997-04-17) claims 1-39; examples 4-5 page 17, lines 31-32	1-21
A	WO 2015/177379 A2 (REPONEX PHARMACEUTICALS APS [DK]) 26 November 2015 (2015-11-26) abstract; claims 1-2, 26-31	1-21

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/067428

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 20130020760 A	28-02-2013	KR 20100116537 A	01-11-2010
		KR 20130020760 A	28-02-2013
WO 9305799 A1	01-04-1993	NONE	
WO 02069999 A1	12-09-2002	AT 362375 T	15-06-2007
		DE 60220149 T2	13-12-2007
		DK 1372700 T3	24-09-2007
		EP 1372700 A1	02-01-2004
		ES 2287296 T3	16-12-2007
		JP 2004536038 A	02-12-2004
		JP 2009161538 A	23-07-2009
		PT 1372700 E	27-08-2007
		US 2002141970 A1	03-10-2002
		US 2007041938 A1	22-02-2007
		US 2008299071 A1	04-12-2008
		WO 02069999 A1	12-09-2002
EP 1598074 A1	23-11-2005	AU 2004216298 A1	10-09-2004
		CA 2517310 A1	10-09-2004
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		JP 4607010 B2	05-01-2011
		JP 5377431 B2	25-12-2013
		JP 2010215664 A	30-09-2010
		JP WO2004075913 A1	01-06-2006
		KR 20050113199 A	01-12-2005
		US 2006159653 A1	20-07-2006
		US 2009264629 A1	22-10-2009
		WO 2004075913 A1	10-09-2004
WO 2008122415 A1	16-10-2008	AT 493117 T	15-01-2011
		AU 2008235044 A1	16-10-2008
		CA 2679551 A1	16-10-2008
		CN 101657187 A	24-02-2010
		EP 2142174 A1	13-01-2010
		HK 1135329 A1	15-04-2011
		JP 5349452 B2	20-11-2013
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