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Stable lyophilized pharmaceutical preparations of G-CSF

The present invention concerns lyophilized pharmaceutical preparations of G-CSF which contain maltose, raffinose, sucrose, trehalose or amino sugars as stabilizers. In addition the invention concerns a process for the production of these stabilized lyophilisates and the use of maltose, raffinose, sucrose, trehalose or amino sugars as stabilizers of pharmaceutical agents containing G-CSF.

Various pharmaceutical preparations which contain G-CSF (granulocyte colony stimulating factor) are already known from the state of the art.

A pharmaceutical agent containing G-CSF is described in DE 37 23 781 (GB 2,193,631) which contains at least one pharmaceutically acceptable surface-active agent, saccharide, protein or a high-molecular compound for stabilizing G-CSF. Preparations are proposed there which contain human serum albumin as a stabilizing agent. In particular preparations are stated to be advantageous which contain surface-active agents in parts by weight which correspond to 1 - 10000-fold the amount of G-CSF used.

Stabilized preparations of G-CSF are described in EP 0 373 679 which are characterized essentially by an acidic pH value of the solution, wherein the solutions should have as low a conductivity as possible. The

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solutions have a pH value of 3 - 3.7 in the case that the solutions contain further pharmaceutical auxiliary substances such as for example buffers or mannitol. If no buffer substances are present in the pharmaceutical formulation, pH ranges of 2.75 - 4 are described as being advantageous.

Furthermore, stabilized lyophilisates of human protein preparations are described in EP 0 306 824 in which stabilization is achieved by adding a mixture of urea, amino acids and detergent.

In the earlier PCT Patent Application PCT/EP92/01823 a process is described for the production of well-tolerated pharmaceutical agents containing G-CSF for infusion or injection purposes. The liquid forms of administration are characterized in particular by a low titration acidity and low buffer capacity. The pH values of the described infusion and injection solutions containing G-CSF are in an acidic range of about 3.8 - 4.5.

Processes for the production of liquid drug administration forms containing G-CSF which additionally contain preservatives are known from PCT/EP92/01822. The pH values of the aqueous pharmaceutical solutions are in the acidic range of 2.5 - 4.5. In this case the stabilization of G-CSF is essentially achieved by setting an acidic pH value which is favourable for G-CSF and by adding a mixture of various amino acids.

The previously known drug administration forms for G-CSF do, however, have some disadvantages. It was found that liquid G-CSF preparations can in some cases be sensitive

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towards freezing and thawing. The uncontrolled freezing and thawing of such preparations can lead to the formation of dimers, oligomers and aggregates; insoluble precipitates may also be formed. Such properties of pharmaceutical agents containing protein are questionable from a medical-pharmaceutical point of view since it is not possible to absolutely avoid an accidental freezing of the pharmaceutical solution and thus there is a risk of administering a qualitatively changed preparation.

A disadvantage of the preparations described in DE 37 23 781 is moreover that they contain pharmaceutical additives or auxiliary substances which, from a medical point of view, cannot simply be judged as harmless. Polymers and proteins have a certain potential risk with regard to their suitability as pharmaceutical additives due to their origin and their physicochemical properties. Proteins of human or animal origin as well as proteins obtained from cell cultures, bear a potential residual risk of viral contaminations. Other protein-like impurities which are analytically difficult to detect can also cause immunological reactions in humans due to their antigenic properties. Moreover proteins of animal origin can generally trigger immunological reactions in humans due to their species-specific properties. Long-term reactions after a later re-administration of such proteins are also possible.

The addition of high-molecular compounds (polymers) may also be problematic. Polymers can accumulate in the body due to their large molecular mass and can thus remain in the body over a long period if no biodegradation occurs. This is a particular risk in the case of subcutaneous administration since removal and distribution via the

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blood stream is considerably slower compared to intravenous administration. Polymers may also have antigenic properties depending on their molecular mass. In addition the purity of polymers is difficult to guarantee because of the catalysts used for their production or the presence of monomers and other polymer fragments. The use of polymers in pharmaceutical forms of administration should thus be avoided if possible in particular in the case of forms of pharmaceutical agents that can be administered subcutaneously.

The amounts of surfactant described in DE 37 23 781 must also be regarded as problematic from a medical point of view. Here surfactant concentrations are described as being advantageous in which 1 to 10000 parts by weight of a surface-active agent are present in relation to the proportion by weight of G-CSF. If, on the other hand, one considers the preferred application concentrations of
G-CSF for clinical use of 0.05 - 1.5 mg/ml in the final pharmaceutical formulations, then this results in correspondingly high surfactant concentrations. These should be avoided from a medical point of view since they can cause local irritations.

In addition some of the known formulations have the disadvantage that, particularly in the case of subcutaneous administration, they lead to local intolerances in patients due to the low pH value used. The product obtained can cause pain and local tissue irritation in sensitive patients since the physiological pH range of 7.0 - 7.5 present in tissue is not adhered to.

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In addition it is known from the literature that especially non-glycosylated forms of G-CSF are particuarly unstable compared to glycosylated G-CSF which is obtained from CHO cells (J. Biol. Chem. 1990, 265, 11432). The stabilization of non-glycosylated forms of G-CSF proved to be particularly difficult and requires specially selected measures in order to formulate this molecule in a stable drug administration form.

The object of the present invention was to provide a drug administration form of G-CSF which enables a proper use of G-CSF as a pharmaceutical agent and which does not have the disadvantages of the previously known drug forms described above. The pharmaceutical preparation should be stable towards uncontrolled freezing and thawing processes as well as also being stable when stored for long periods as a lyophilisate, physiologically well-tolerated, simple to use and possible to dose precisely.

The examples described in DE 37 23 781 show that stable lyophilisates can be obtained when human serum albumin is used as an auxiliary agent. The addition of sugar alcohols alone leads to less stable formulations. Therefore with regard to improving the state of the art it is desirable to find formulations which do not contain human serum albumin (HSA) or other proteins or polymers but nevertheless have a good stability even at increased temperatures. The absence of human serum albumin and polymers reduces the medical risk of side-effects such as those described for example for HSA.

Surprisingly it was found that, within the sense of the

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present invention, it is possible to produce stable forms of pharmaceutical agents when maltose, raffinose, sucrose, trehalose or amino sugars are used as additives.

Solid preparations which contain maltose, raffinose, sucrose, trehalose or amino sugars as auxiliary agents can be frozen or even stored at increased temperatures (up to 40°C) with no significant loss of protein quality. The pharmaceutical quality of the active substance is not adversely affected by this. The preparations according to the invention are preferably put on the market as lyophilisates. The aqueous preparations prepared after redissolving are very well-tolerated and represent high quality preparations with regard to protein stability. In addition they have the advantage that the addition of maltose, raffinose, sucrose, trehalose or amino sugars as auxiliary agents enables solutions to be prepared with an advantageous pH value of 4 - 5 or 7 - 8 whereas the solutions known from the state of the art in general require solutions with a pH value of 2.5 - 3.5 for stabilizing the protein.

An additional advantage of the preparations according to the invention is that they are essentially free of protein-like or polymeric auxiliary substances the use of which may be problematic from a medical point of view. Due to the fact that liquid drug administration forms containing G-CSF obtained by dissolving lyophilisates can now be produced with a pH value of about 4 - 5 or 7 - 8, preferably with a pH value near to the pH value of blood (pH 7.2 - 7.4), they also have the advantage of being well tolerated and capable of administration substantially free of pain. This is particularly important for subcutaneous administration

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since in this case intolerances occur more easily than with intravenous administration. The preparations according to the invention can also be prepared in the clinically particularly preferred concentration ranges of 0.05 - 1.5 mg/ml so that it is possible to keep to injection volumes of $\leq$ 1.0 ml. Small injection volumes are particularly advantageous for subcutaneous administration since they cause only slight mechanical stimuli in the hypodermis.

A further advantage is that due to the selected auxiliary substances, the relatively high amounts of surfactant previously required in the liquid pharmaceutical preparations are no longer necessary. On the contrary low amounts of surfactant of 0.5 mg/ml or less, preferably of 0.01 - 0.1 mg/ml, are adequate to stabilize G-CSF. Surfactant concentrations (mg/ml) can be used advantageously which are smaller than or at most the same as the amount of G-CSF protein used per unit of volume (mg/ml). This is a particular advantage for those liquid drug forms which are intended for the subcutaneous administration of G-CSF. In addition the measures according to the invention lead to an adequate stabilization for pharmaceutical preparations of the labile non-glycosylated G-CSF molecules in particular.

The auxiliary agent maltose (malt sugar, maltobiose, 4-O-alpha-D-glucopyranosyl-D-glucose) is used in an amount of 0.01 - 10000-fold the amount of the active substance G-CSF. The same applies to the auxiliary substances raffinose, sucrose and trehalose. The concentration of these auxiliary substances in the liquid form of the pharmaceutical agent is 0.1 - 200 mg/ml, preferably 10 - 60 mg/ml. The stereoisomeric disaccharides cellobiose, gentiobiose or isomaltose can also be used instead of

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maltose. Amino sugars generally denote those monosaccharides which have an amino or an acylated amino group instead of a hydroxy group. Examples of this are glucosamine, galactosamine and neuraminic acid.

In a particular embodiment pharmaceutical preparations are provided which contain amino acids in addition to maltose, raffinose, sucrose or trehalose. Basic amino acids come into particular consideration as amino acids such as for example arginine, lysine, ornithine etc., acidic amino acids such as for example glutamic acid, aspartic acid etc. and also aromatic amino acids such as phenylalanine, tyrosine, tryptophan etc.

Amino acids are used in 0.01 - 10000-fold the amount of the active substance G-CSF. The concentration of these auxiliary substances in the liquid pharmaceutical preparation is 0.1 - 200 mg/ml, preferably 1 - 50 mg/ml.

In order to produce the lyophilisates, firstly the aqueous pharmaceutical solutions which contain the active substance and other common pharmaceutical auxiliary sustances are prepared. Amino acids such as e.g arginine, lysine, ornithine, phenylalanine or tyrosine come in particular into consideration as pharmaceutical auxiliary substances. In addition the aqueous preparation can contain common buffer substances such as e. g. acetic acid, hydrochloric acid, citric acid, lactic acid, tartaric acid, maleic acid and phosphoric acid or physiologically tolerated salts thereof. In the production of the auxiliary substance solution these buffer substances can either be present in the form of the corresponding free acids or in the form of the alkali, alkaline-earth or ammonium salts.

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The solution can in addition contain further common pharmaceutical auxiliary substances.

The sequence of addition of the various auxiliary substances or of the active substance is largely independent of the production process and is at the discretion of the person skilled in the art. The desired pH value of the solution is adjusted by adding bases such as alkali hydroxides, alkaline-earth hydroxides or ammonium hydroxide. Sodium hydroxide is preferably used for this. The adjustment of the desired pH value can in principle be achieved by adding basic solutions. In general salts of strong bases with weak acids come into consideration for this such as e.g. sodium acetate, sodium citrate, di-sodium or di-potassium hydrogen phosphate or sodium carbonate. If the pharmaceutical solution of auxiliary substance has a basic pH value, it is adjusted by titration with an acid until the desired pH range is reached. Physiologically tolerated inorganic or organic acids come into consideration as acids such as for example hydrochloric acid, phosphoric acid, acetic acid, citric acid or conventional solutions of substances which have an acidic pH value. In this respect preferred substances are salts of strong acids with weak bases such as e.g. sodium dihydrogen phosphate or potassium dihydrogen phosphate.

The concentrations of the buffer substances in the ready-to-administer liquid drug administration form are preferably about 2 - 80 mmol/l in each case. The total concentration of buffer substances should not exceed a value of 100 mmol/l. The concentration of buffer substances is preferably 5 - 40 mmol/l.

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The stabilization of G-CSF molecules by means of the said auxiliary substances relates in principle to all G-CSF molecules produced by recombinant processes and variants thereof. The term G-CSF or G-CSF variant according to the present invention includes all naturally occurring variants of G-CSF as well as G-CSF proteins modified by recombinant DNA technology that are derived therefrom, in particular fusion proteins which additionally contain other protein sequences in addition to the G-CSF part. In this regard a G-CSF mutein is particularly preferred with a N-terminal Met residue at position -1 which is suitable for expression in prokaryotic cells. A recombinant methionine-free G-CSF variant which can be produced according to PCT/EP91/00192 is equally suitable. The term "G-CSF variant" is understood to include those G-CSF molecules in which one or several amino acids may be deleted or replaced by other amino acids wherein the essential properties of G-CSF are substantially retained. Suitable G-CSF muteins are described for example in EP 0 456 200.

It is expedient to add auxiliary substances which act isotonically for the production of well-tolerated parenteral drug administration forms if isotonicity cannot be already achieved by the osmotic properties of the active substance and the auxiliary substances used for stabilization. For this purpose non-ionised, well-tolerated auxiliary substances are mainly used.

It is not advantageous to add salts to adjust the isotonicity, since high concentrations of salts or ions promote the formation of G-CSF aggregates. Therefore salts are advantageously added in small amounts.

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The pharmaceutical preparations can also contain further convential auxiliary substances or additives. Antioxidants such as for example glutathione, ascorbic acid or similar substances, chaotropic auxiliary substances such as for example urea and amino acids such as for example arginine, lysine, ornithine, glutamic acid and others can be added.

The invention is described in more detail in the following on the basis of representative examples of embodiments:

Examples 1 - 14 show in which manner lyophilisates according to the invention can be formulated, manufactured and examined in more detail with regard to the stability of the protein. The influence of the auxiliary substances added in addition to maltose, raffinose, sucrose or trehalose and of the pH value is elucidated.

Comparative experiments on lyophilisates produced on the basis of mannitol or glycine show that maltose, raffinose, sucrose or trehalose lyophilisates produce significantly better results than preparations prepared with other builders. Use of the lyophilisates described according to the invention and elucidated in the examples enables an optimal formulation to be produced for the described objective, which has a physiologically tolerated pH value, has a long-term storage stability enduring increased storage temperatures as well as mechanical stress without negative effects on the protein. The preparations are in particular not sensitive to freezing and it is possible to completely dispense with auxiliary substances that are regarded as

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being critical such as proteins or polymers. In addition they contain only relatively small amounts of physiologically well-tolerated surfactants.

Various sugars or sugar alcohols are examined in example 3 for their stabilizing effect in G-CSF lyophilisates. Maltose turns out to be advantageous compared to lactose and mannitol.

Lyophilisates containing maltose and further auxiliary substances are described in example 4. The results clearly demonstrate that the addition of surfactant does not substantially influence the stability of the preparation, but it prevents the adhesion of the protein to surfaces and thus prevents possible losses in content. The presence of surfactant in such formulations is thus not necessary for reasons of stabilization but rather to maintain the nominal dosage.

Various lyophilisate formulations containing maltose are compared in example 5 with two lyophilisates without maltose which are otherwise formulated identically. The data clearly show that the presence of maltose has an advantageous effect on the examined parameters with respect to stability of the preparation. The addition of further auxiliary substances such as ascorbic acid, glutathione or glutamic acid has no significant influence on the stability within the framework of the examined storage temperatures and storage periods. The preparations described in example 5 are distinguished in particular by the fact that they exhibit no changes in the examined quality criteria on long-term storage at an increased temperature.

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Furthermore it is apparent from the examples that a further buffer salt is not absolutely necessary in lyophilisates which contain maltose and arginine since the arginine buffer formed when the pH is adjusted by hydrochloric acid, phosphoric acid, citric acid or other acids has an adequate pH-stabilizing effect. Arginine buffer is extremely suitable for formulating stable preparations in the pH range under 5.0 and of 7.0 - 7.5 (see examples 11 and 12). Example 9 shows that redissolved lyophilisates with a pH of 7.4 and containing maltose and arginine buffer are stable for at least 24 hours.

G-CSF lyophilisates are described in example 6 which contain amino sugars (galactosamine, N-methyl-glucosamine). It can be seen that the combination of maltose and amino sugar results in more stable preparations than the combination of glycine with amino sugars. This demonstrates that maltose in combination with physiologically well-tolerated auxiliary substances yields considerably more stable and thus higher quality lyophilisates of G-CSF with regard to pharmaceutical quality than other builders and stabilizers proposed in the literature.

Example 7 shows that G-CSF is considerably more stable in lyophilisates containing maltose than in lyophilisates containing mannitol. This is demonstrated accordingly at relevant storage temperatures and for long storage periods.

Example 8 shows that lyophilisates containing maltose at various pH values and containing various auxiliary substance additives give advantageous results compared

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to lyophilisates with other builders and stabilizers (sugar alcohols, amino acids).

Example 10 demonstrates the stability of the lyophilisates according to the invention containing maltose, raffinose, sucrose or trehalose after 13 weeks storage at 40°C.

Example 11 shows that the lyophilisates according to the invention are stable even with higher G-CSF concentrations and the long-term stability of the formulations according to the invention even at increased temperatures is substantiated in example 12.

Example 1:

Test methods for the stability determination

The lyophilized preparations were stored in the dark at defined storage temperatures and subsequently examined with reversed phase HPLC (RP-HPLC), gel chromatography or size exclusion chromatography (SEC HPLC) and Western blot for protein purity as well as for the occurrence of aggregates and dimers. In addition the protein content was examined by OD 280 photometry, the biological activity by bioassay (NFS 60 cell test) and aggregation and precipitation by turbidity measurement. The methods used can be described as follows:

1.1 Reversed phase HPLC

The RP-HPLC was carried out using a Nucleosil C18 column (Knauer Company). The mobile phase consisted

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of 0.12 % (v/v) trifluoroacetic acid (TFA)/water (A) and 0.1 % (v/v) TFA/acetonitrile (B). The chromatography was carried out at a flow rate of 0.5 ml/min using a linear gradient of A to B.

The injection amount was 3 - 6 µg G-CSF depending on the formulation. It was evaluated at a wavelength of 214 nm by means of the peak area using an external standard.

1.2 Size exclusion chromatography (SEC)

A TSK G 2000 SW separation column (7.5 x 300 mm) was used for the SE chromatography. The separations were carried out isocratically at room temperature and a flow rate of 0.6 ml/min in a phosphate buffer (22.2 mM Na_2HPO_4; 107.7 mM KH_2PO_4; pH 6.2). The injected amount was 3 - 6 µg G-CSF. It was evaluated at a detection wavelength of 214 nm by means of the peak area using an external standard.

1.3 SDS page/Western blot

3 µg rhG-CSF is applied under non-reducing conditions to a 12 per cent polyacrylamide SDS gel and subjected to gel electrophoresis. Subsequently the G-CSF monomers, dimers or aggregates separated according to their molecular weight are transferred by electro-blotting onto nitrocellulose. The protein bands are identified by incubation with a specific polyclonal biotinylated anti-G-CSF antibody (PAB < GCSF > IgG) and detected by means of the phosphatase technique using streptavidin-alkaline phosphatase conjugate (SA-AP conjugate),

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5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). The percentage amounts of monomers, dimers and aggregates are determined by laser densitometric evaluation with the aid of a series of rhG-CSF standards.

1.4 NFS-60 bioassay (biological activity)

The in vitro determination of G-CSF activity is based on the measurement of cell counts in a cell culture of NFS-60 cells stimulated by G-CSF.

Under suitable conditions it is possible to correlate the dehydrogenase activity of the cells with the concentration of G-CSF in the medium. Suitable dilutions of the G-CSF buffer solution are prepared in order to obtain a readily measurable increase in the dehydrogenase activity.

The activity is then measured photometrically at 570 and 690 nm; the reduction of the tetrazolium salt MTT (yellow) to formazan (blue) is measured.

The in vitro activity of G-CSF is calculated by comparing the data for the sample with those for the standard according to the parallel line method. The evaluation is according to the requirements of Ph. Eur. (VIII, 13).

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1.5 Scattered light measurement, turbidity measurement

The measurement is carried out directly on the undiluted product solution in glass cuvettes (diameter 2 cm). The scattered light that is diffusely deflected by the liquid is measured at an angle of 90 degrees. It is measured in comparison to formazine standard suspensions according to DIN 38404C2, the values are stated in TU/F. The measurement is carried out on a suitable turbidity photometer, e.g. LTP 5 (Dr. Lange Company, Düsseldorf).

1.6 Photometry OD 280 (protein content)

The G-CSF UV spectrum has an absorbance maximum at 280 nm which is due to side chain chromophores such as tryptophan, tyrosine and phenylalanine residues. The measurement is carried out in comparison to placebo solutions by means of:

- UV spectrophotometer
  (e.g. Uvikon 810 P or 941, Kontron Instruments)

- Semi-micro quartz cuvettes, 500 µl, path length: 1 cm (e.g. Hellma, Suprasil, Cat. No. 104.002B-QS)

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Example 2:

Aqueous solutions of 0.1 mg/1 ml Poloxamer 118 and 50 mg/ml of the following sugars or sugar alcohols, mannitol (formulation 1), lactose (formulation 2) and maltose (formulation 3) were admixed with G-CSF at a concentration of 70 µg/ml. After filtration through a sterilized 0.2 µm membrane filter, the solutions were filled into sterile injection bottles made of glass of hydrolytic class I. After lyophilisation they were gassed with sterile nitrogen and the stoppers which were firstly put on loosely were pressed in under aseptic conditions to close the lyophilisates. The lyophilisates were flanged and stored in the dark for 6 and 13 weeks at different temperatures. Afterwards the stability of the preparation was examined with the methods described below.

Table 1a: Storage at 20°C

	6 weeks storage at 20°C			13 weeks storage at 20°C		
	I	II	III	I	II	III
Formul. 1 mannitol	>99%	91%	36%	86%	47%	27%
Formul. 2 lactose	>99%	>99%	18%	>99%	>99%	12%
Formul. 3 maltose	>99%	>99%	6%	>99%	>99%	7.8%

I Purity of unchanged protein in RP HPLC
II Purity of unchanged protein in SEC HPLC
III Dimers/aggregates in Western blot

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Table 1b: Storage at 40°C

	6 weeks storage at 40°C			13 weeks storage at 40°C		
	I	II	III	I	II	III
Formul. 1 mannitol	81%	70%	50%	69%	25%	70%
Formul. 2 lactose	>99%	>99%	37%	>99%	>99%	not detectable/ aggregated
Formul. 3 maltose	>99%	>99%	6.4%	>99%	>99%	12%

I Purity of unchanged protein in RP HPLC
II Purity of unchanged protein in SEC HPLC
III Dimers/aggregates in Western blot

Example 3:

Lyophilisates of G-CSF were prepared. For this the auxiliary substances stated in the following table were dissolved in water for injection purposes, subsequently G-CSF was added at a concentration of 70 µg/ml and if necessary the pH value was adjusted exactly with small amounts of the buffer system. Pluronic F 68 was used as a representative of an appropriate surfactant. Other surfactants behave similarly. After sterilization by filtration through a suitable 0.2 µm membrane filter, the solutions were filled into sterile injection bottles made of glass of hydrolytic class I and lyophilized according to conventional methods. After lyophilisation they were aerated with nitrogen and the injection bottles were closed under aseptic conditions with freeze-drying stoppers. The preparations were stored in flanged bottles in the dark at defined storage

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temperatures for 6 and 12 weeks and examined with the methods stated in example 1.

Table 2: Formulations of G-CSF at pH 3.6 containing maltose

	Formulation 4	Formulation 5
G-CSF	70 µg	70 µg
Maltose	35 mg	35 mg
L-Phenylalanine	10 mg	10 mg
Ascorbic acid	5 mg	5 mg
Glutathione	10 mg	10 mg
L-Glutamic acid	5 mg	5 mg
L-Arginine	10 mg	10 mg
Buffer (pH)	to pH 3.6	to pH 3.6
Pluronic F68	-	0.1 mg
Water for injection purposes	up to 1 ml	up to 1 ml

Table 3: Storage at 20°C

Fm.	Storage temp.	after 6 weeks aggr. in %	after 12 weeks aggr. in %	after 6 weeks SEC %G-CSF	after 6 weeks RP %G-CSF	after 12 weeks SEC %G-CSF	after 12 weeks RP %G-CSF
4	+20°C	0.0	2.6	61	63	64	69
5	+20°C	0.0	0.5	>99	>99	>99	>99

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Example 4:

G-CSF lyophilisates containing 500 µg/ml G-CSF (formulations 6 - 10) were prepared as follows. The auxiliary substances stated in the following table were dissolved in water for injection purposes, G-CSF was added and if necessary the pH value was adjusted with small amounts of hydrochloric acid or disodium hydrogen phosphate. In each case 1 ml of the solutions that were previously sterilized by filtration through a 0.2 µm membrane filter were filled into injection bottles made of glass of hydrolytic glass I and freeze-dried according to conventional methods. After the lyophilisation they were aerated with nitrogen and the lyophilisates were closed under aseptic conditions with freeze-drying stoppers. The flanged lyophilisates were stored in the dark at defined temperatures and examined using the methods stated in example 1.

Table 4: Compositions of formulations 6 - 10

	Formul.6	Formul.7	Formul.8	Formul.9	Formul.10
G-CSF	0.5 µg	0.5 µg	0.5 µg	0.5 µg	0.5 µg
Maltose	35 mg	35 mg	35 mg	-	-
L-Phenylalanine	10 mg	10 mg	10 mg	10 mg	10 mg
Ascorbic acid	5 mg	-	-	5 mg	-
Glutathione	10 mg	-	-	10 mg	-
L-Glutamic acid	5 mg	-	-	5 mg	-
L-Arginine	10 mg	10 mg	10 mg	10 mg	10 mg
Buffer (pH)	to 4.5	to 4.5	to 6.5	to 4.5	to 6.5
Pluronic F68	0.1 mg	0.1 mg	0.1 mg	0.1 mg	0.1 mg
Water for injection purposes	to 1 ml	to 1 ml	to 1 ml	to 1 ml	to 1 ml

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Table 5: Analytical results

Form	Storage temp.	Western blot 6 w %aggr.	Western blot 12 w %aggr.	after 6 weeks SEC %G-CSF	after 6 weeks SEC %aggr.	after 6 weeks RP %G-CSF	after 13 weeks SEC %G-CSF	after 13 weeks SEC %aggr.	after 13 weeks RP %G-CSF
6	+ 8°C	< 1	1.0	>99%	0.9	>99%	>99%	0.7	99%
	+ 40°C	< 1	1.7	>99%	0.6	>99%	>99%	0.6	98%
7	+ 8°C	< 1	1.1	>99%	1.6	>99%	>99%	1.1	99%
	+ 40°C	< 1	2.3	>99%	1.9	>99%	>99%	1.1	99%
8	+ 8°C	< 1	-				>98%	1.5	>99%
	+ 40°C	< 1	-				>98%	1.4	>99%
9	+ 8°C	3.8	0.3	95%	5.8	>99%	95%	1.5	98%
	+ 40°C	7.9	2.5	95%	6.4	93%	86%	0.8	94%
10	+ 8°C	-	5.2				96%	1.0	95%
	+ 40°C	-	10.3				89%	2.6	89%

Example 5:

The formulations stated in the following table (formulations 11 - 14) were prepared as follows: the auxiliary substances were dissolved in water for injection purposes, subsequently G-CSF was added at the stated concentration. If necessary the pH value was adjusted exactly with the aid of components of the phosphate buffer. The solutions were then filtered through a sterilized membrane filter with a pore size of 0.2 µm and filled under aseptic conditions into injection bottles of hydrolytic class I and lyophilized. After lyophilisation they were aerated with nitrogen, the lyophilisates were closed under aseptic conditions with freeze-drying rubber stoppers and flanged. The

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lyophilisates were stressed in the dark at defined storage temperatures. After 6 and 13 weeks they were examined with the methods stated in example 1.

Table 6: Lyophilisate preparations containing amino sugars

	Form. 11	Form. 12	Form. 13	Form. 14
G-CSF	0.5 µg	0.5 µg	0.5 µg	0.5 µg
Pluronic F68	0.1 mg	0.1 mg	0.1 mg	0.1 mg
N-methyl-glucosamine	-	10 mg	-	10 mg
Galactosamine	10 mg	-	10 mg	-
Glycine	-	-	35 mg	35 mg
Maltose	35 mg	35 mg	-	-
Phenylalanine	10 mg	-	-	-
Phosphate buffer	to pH 7.0	to pH 7.0	to pH 7.0	to pH 7.0
Water for injection purposes	up to 1.0 ml	up to 1.0 ml	up to 1.0 ml	up to 1.0 ml

The analytical data obtained after storage of the aforementioned preparations are summarized in the following results table.

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Table 7: Analytical results
DCP = decomposition products

Form.	Storage	6 Weeks West. blot % aggr.	12 Weeks West. blot % aggr.	12 Weeks RP-HPLC % G-CSF	12 Weeks % DCP in SEC HPLC
11	+ 8°C	3.8	2.9	>99	1.2
	+ 40°C	3.2	2.3	>99	1.8
12	+ 8°C	1.8	3.8	>99	1.4
	+ 40°C	1.7	4.5	>99	0.7
13	+ 8°C	1.1	1.4	>99	0.9
	+ 40°C	16.8	13.0	75	4.2
14	+ 8°C	1.6	12.4	97.5	1.2
	+ 40°C	7.7	26.3	84.5	3.5

Example 6:

Formulations 15 and 16 described in the following were prepared as follows: The stated auxiliary substances were dissolved in water for injection purposes, G-CSF was added in the stated concentration. The pH was adjusted if necessary using portions of buffer components. Afterwards the solution was filtered through a sterile membrane filter with a pore size of 0.2 µm and dispensed under aseptic conditions into sterile injection bottles made of glass of hydrolytic class I. Subsequently the injection preparations were freeze-dried, then aerated with nitrogen and the injection bottles were closed under aseptic conditions with freeze-drying stoppers and subsequently flanged. The formulations were stored in the dark at defined temperatures and examined for the parameters mentioned below. The test methods stated in example 1 were used for this.

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Formulation 15

G-CSF	**0.25**
Polysorbate 80	0.05 mg
Phenylalanine	5 mg
Maltose	17.5 mg
L-arginine	5 mg
Buffer	to pH 4.5
Water for injection purposes	up to 0.5 ml

Formulation 16

G-CSF	**0.3 mg**
Polysorbate 80	0.1 mg
Mannitol	50 mg
Buffer	to pH 4.5
Water for injection purposes	up to 1.0 ml

Table 8: Examination results after storage of formulations 15 and 16 for 3 and 6 months

	Formulation 15				Formulation 16			
	storage period 3 months		storage period 6 months		storage period 3 months		storage period 6 months	
	4-8°C	23°C	4-8°C	23°C	4-8°C	23°C	4-8°C	23°C
West.blot (dimers)	2.2	< 1%	1.3%	0.7	< 1%	12%	4.1%	17
SEC-HPLC (dimers)	< 1%	<1%	< 1%	< 1%	< 1%	2%	< 1%	3
RP-HPLC (G-CSF peak)	>99%	>99%	>98%	>98%	>99%	98.2%	>98%	>98

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Example 7:

The formulations described in table 9 were prepared as follows:

The stated auxiliary substances were dissolved in water for injection purposes, G-CSF was added in the stated concentration, afterwards the pH was adjusted if necessary with small portions of buffer components. The pharmaceutical solution was then filtered through a sterilized membrane filter with a pore size of 0.2 µm and subsequently dispensed into sterile injection bottles made of glass of hydrolytic class I under aseptic conditions and lyophilized.

After lyophilisation they were aerated with nitrogen and the bottles were closed under aseptic conditions with freeze-drying stoppers. The bottles were flanged and stored in the dark under defined temperature conditions. After the respective storage periods, analytical examinations were carried out using the methods described in example 1 (cf. table 10).

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Table 9: G-CSF lyophilisates containing mannitol compared to other builders

	Form. 17	Form. 18	Form. 19	Form. 20	Form. 21	Form. 22	Form. 23	Form. 24
G-CSF	350 µg	350 µg	175 µg	175 µg	175 µg	175 µg	175 µg	175 µg
Maltose			17.5 mg	17.5 mg	17.5 mg	-	-	-
Glycine						4 mg	10 mg	8.9 mg
Arginine			5 mg	-	5 mg	5 mg	-	
Phenylalanine			5 mg	5 mg	5 mg	5 mg	-	2.5 mg
Mannitol	50 mg	50 mg				-	-	
Tween 80	0.1 mg	0.1 mg	0.05 mg	0.05 mg	0.05 mg	0.05 mg	0.05 mg	0.05 mg
pH	4.5	7.2	4.5	7.2	7.2	7.2	7.2	7.2
(buffer)	HCl	phosphate	HCl	HCl	HCl	HCl	phosphate	phosphate
Water for injection purposes	up to 1.0 ml	up to 1.0 ml	up to 0.5 ml	up to 0.5 ml	up to 0.5 ml	up to 0.5 ml	up to 0.5 ml	up to 0.5 ml

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In Table 10 the analytical data are summarized for the stated formulations:

Formulation	Storage	4 weeks RP-HPLC % G-CSF	4 weeks SEC-HPLC % G-CSF	4 weeks Western blot
17	8°C	> 99	> 99	3.6 % dimers
	30°C	94	92	9.6 % dimers
	40°C			14.4 % dimers
18	8°C	69	60	aggregates
	30°C	44	36	aggregates
	40°C	13	12	aggregates
19	8°C	> 99	> 99	1.0 % dimers
	30°C	> 99	95.5	0.5 % dimers
	40°C	> 99	97.5	0.5 % dimers
20	8°C	> 99	> 99	1.6 % dimers
	30°C	> 99	> 99	1,4 % dimers
	40°C	> 99	> 99	2.3 % dimers
21	8°C	> 99	> 99	1.5 % dimers
	30°C	> 99	97.5	2.1 % dimers
	40°C	> 99	97	2.0 % dimers
22	8°C	> 99	> 99	2.8% di/aggr.
	30°C	96	96	3.0% di/aggr.
	40°C			12 % di/aggr.
23	8°C	> 99	> 99	6.8 % dimers
	30°C	91.5	92	aggregates
	40°C	79	74	aggregates
24	8°C	> 99	> 99	10.8 % dimers
	30°C	88	85	aggregates
	40°C	67	60	aggregates

Example 8:

Standing time of redissolved lyophilisates according to the invention having a pH of 7.4

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The following composition was prepared:

mg/ml	Formulation 25
G-CSF	0.35
Polysorbate 80	0.1
Maltose	50
Arginine	10
Phenylalanine	10
Hydrochloric acid	to pH 7.4

The stated auxiliary substances were dissolved in 1 ml water for injection purposes, G-CSF was added and the pH value was adjusted to pH 7.4. The solution was sterilized by filtration through a sterilized membrane filter with a pore size of 0.2 µm and afterwards dispensed into injection bottles of hydrolytic class I.

After putting on suitable freeze-drying stoppers, the preparation was freeze-dried at a main drying temperature of -25°C and a subsequent drying temperature of +8°C until the residual moisture reached < 5 %. The dried lyophilisates were aerated with nitrogen and closed.

After 6 months storage at 4 - 8°C the lyophilisates were dissolved in 1 ml water for injection purposes and allowed to stand for 24 hours at room temperature.

After this standing time there was no change using the examination methods described in example 1 with regard to biological activity (NFS-60 test), protein content (photometry OD 280) and purity (Western blot), purity (SEC HPLC), purity (SDS page) and purity (RP HPLC) compared to samples examined directly after dissolution. Turbidity measurements - even under

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mechanical stress - resulted in very low turbidity values.

This clearly indicates that redissolved lyophilisates of the formulation according to the invention containing maltose and arginine buffer at pH 7.4 have an adequate standing time for clinical application.

Example 9:

Stability of lyophilisates according to the invention containing maltose, raffinose, sucrose or trehalose after 13 weeks storage at 40°C

Three lyophilisates corresponding to example 8, formulation 25, were prepared that contained 50 mg/ml maltose or the same amount by weight of a) raffinose or b) sucrose or c) trehalose.

All lyophilisates were stored for 13 weeks at temperatures of 5°C, 25°C, 30°C and 40°C, afterwards they were dissolved and examined visually and using the examination methods SEC HPLC, RP HPLC, Western blot and SDS page described in example 1.

In all cases clear colourless solutions resulted. In SEC HPLC the product peaks had a size of > 98 % and the dimers/aggregates had a size of < 1%. In RP HPLC the product peak reached 100 %, secondary peaks could not be detected, the main peak corresponded to the working standard. In SDS-page no degradation products, dimers or aggregates could be detected.

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Table: after 13 weeks storage period

Temp.	SEC HPLC % G-CSF	RP HPLC % G-CSF	Western blot % aggregates	SDS-Page % minor bands
5°C	>98%	100%	n.d.	<1
20°C	>98%	100%	n.d.	<1
30°C	>98%	100%	n.d.	<1
40°C	>98%	100%	n.d.	<1

Example 10:

Stability of maltose lyophilisates according to the invention containing arginine phosphate and arginine chloride buffers at pH 4.5 and pH 7.2 after 13 weeks storage at 30°C

The following formulations were prepared according to the production process described in example 8, which only differed in their buffers and pH values:

	Formulation 26	Formulation 27	Formulation 28	Formulation 29
G-CSF	0.35 mg	0.35 mg	0.35 mg	0.35 mg
Polysorbate 80	0.1 mg	0.1 mg	0.1 mg	0.1 mg
Phenylalanine	10 mg	10 mg	10 mg	10 mg
Arginine	10 mg	10 mg	10 mg	10 mg
Maltose	47.5 mg	47.5 mg	47.5 mg	47.5 mg
Phosphoric acid	to pH 4.5	to pH 7.2		
Hydrochloric acid			to pH 4.5	to pH 7.2

The preparations were stored at temperatures of 4 to 8°C, 20 - 25°C and 30°C, dissolved after 13 weeks in 1 ml water for injection purposes and examined using the examination methods described in example 1 i.e. RP HPLC, SEC HPLC (purity) and Western blot (degradation, dimerization and aggregate formation). The results are shown in table 11 and show that the lyophilisates at pH 4.5 and 7.2 according to the invention are stable after 13 weeks storage at 30°C.

Table 11: Results after 13 weeks at 30°C

	RP-HPLC	SEC-HPLC	Western blot		
	% minor peaks		degrad.	dimers	aggregate
Formulation 26	<1	<1	n.d.	n.d.	n.d.
Formulation 27	<1	<1	n.d.	n.d.	n.d.
Formulation 28	<1	<1	n.d.	<1%	n.d.
Formulation 29	<1	<1	n.d.	<1%	n.d.

n.d. = not detectable

Example 11:

Stability of maltose lyophilisates according to the invention containing arginine phosphate and arginine chloride buffer at pH 7.4 after 4 and 13 weeks storage at 40°C

Formulations were prepared in the same manner as described in example 8 and their pH value was adjusted to 7.4 in one case with hydrochloric acid and once with

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phosphoric acid.

	Formulation 30	Formulation 31
G-CSF	0.35 mg	0.35 mg
Polysorbate 80	0.1 mg	0.1 mg
Phenylalanine	10 mg	10 mg
Arginine	10 mg	10 mg
Maltose	47.5 mg	47.5 mg
Phosphoric acid	pH 7.4	
Hydrochloric acid		pH 7.4

These two preparations were stored for 4 and 13 weeks at temperatures of 4 - 8°C and 40°C. The examination results (Western blot, SDS page) after 13 weeks storage are shown in the following table 12. The results after 4 weeks storage are identical.

The results show that the maltose lyophilisates at pH 7.4 according to the invention are stable after 13 weeks storage at 40°C.

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Table 12: Results after 13 weeks at 40°C

	Storage temp.	Western blot, non-reduc.		SDS-Page, reduc.			Residual moisture %
		aggr. <1%	dimers <2%	product band	degr. prod.	other bands	
Form. 30	5°C	n.d.	n.d.	<98%	<1%	n.d.	1.4
	40°C	n.d.	n.d.	<98%	<1%	n.d.	2.0
Form. 31	5°C	n.d.	n.d.	<98%	<1%	n.d.	1.9
	40°C	n.d.	n.d.	<98%	<1%	n.d.	2.1

Example 12:

Stability of lyophilisates according to the invention having a pH of 7.4 and G-CSF concentrations of 0.5 and 1.0 mg/ml after 13 weeks storage at 40°C

The following formulations were prepared according to the production process described in example 8 which differ in their content of G-CSF:

mg/ml	Formulation 32	Formulation 33
G-CSF	0.5 mg	1.0 mg
Polysorbate 80	0.1 mg	0.1 mg
Phenylalanine	10 mg	10 mg
Arginine	10 mg	10 mg
Maltose	47.5 mg	47.5 mg
Phosphoric acid	pH 7.4	pH 7.4

The formulations were stored for 4 weeks and 13 weeks at -20°C, 4 - 8°C, 20°C - 25°C, 30°C and 40°C, afterwards dissolved in 1 ml water for injection purposes and

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examined using the examination methods SEC HPLC, RP HPLC, Western blot and SDS-page described in example 1 (examination results see table 13).

The results show that the lyophilisates of the formulations according to the invention are stable even at a higher protein concentration of up to 1 mg/ml after 13 weeks storage at 40°C.

Example 14:

Long-term stability during 9 months

Lyophilisates were prepared using formulation 31 of example 11 and the preparations were stored for 9 months at temperatures of -20°C, 5°C, 25°C, 30°C and 40°C and examined after 3, 6 and 9 months using all examination methods described in example 1.

No change could be detected in any of the examined parameters during the storage period. At the end of the storage periods and at all temperatures the preparation proved to be fully biologically active, it had the complete protein content and showed bands or peaks in all determinations of purity which were well below 1 % of the intact G-CSF molecule.

The results show that lyophilisates according to the invention are stable even when stored for long periods at higher temperatures and thus greatly exceed the stabilites described in the state of the art.

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Table 14: Storage at 30°C

	3 months	6 months	9 months
NFS 60 test 80 - 125%	corresponds	corresponds	corresponds
OD 280	358 mg	360 mg	352 mg
SDS page minor bands	<1%	<1%	<1%
Western blot % aggregates %dimers	n.d. <1%	n.d. <1%	n.d. <1%

	3 months	6 months	9 months
RP HPLC Product peak	>99%	>99%	>99%
SEC HPLC Product peak Minor peaks	>98% n.d.	>98% n.d.	>98% n.d.
Turbidity measurement TU/F	0.5	0.5	0.5

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Table 13:

Formulation 32

Test parameter	0 weeks [refrigerator]	13 weeks [-20°C]	13 weeks [refrigerator]	13 weeks [RT]	13 weeks [30°C]	13 weeks [40°C]
Visual examination						
-appearance, lyophilisate	-	white	white	white	white	white
-clarity, (solution)	-	clear	clear	clear	clear	clear
SEC-HPLC						
[purity %]	>98	>98	>98	>98	>98	>98
[dimers/aggregates %]	<1	<1	<1	<1	<1	<1
RP-HPLC						
[purity %]	>98	>98	>98	>98	>98	>98
[sum of minor peaks %]	<1	<1	<1	<1	<1	<1
Western blot						
[dimers %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[aggregates %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[degradation products %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SDS-PAGE, silver stain						
[monomers %]	-	>>99%	>>99%	>>99%	>>99%	>>99%
[additional bands %]	-	n.d.	n.d.	n.d.	n.d.	n.d.
[degradation products %]	-	<<1%	<<1%	<<1%	<<1%	<<1%

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Formulation 33

Test parameter	0 weeks [refrigerator]	13 weeks [-20°C]	13 weeks [refrigerator]	13 weeks [RT]	13 weeks [30°C]	13 weeks [40°C]
Visual examination						
-appearance, lyophilisate	-	white	white	white	white	white
-clarity, (solution)	-	clear	clear	clear	clear	clear
SEC-HPLC						
[purity %]	>98	>98	>98	>98	>98	>98
[dimers/aggregates %]	<1	<1	<1	<1	<1	<1
RP-HPLC						
[purity %]	>98	>98	>98	>98	>98	>98
[sum of minor peaks %]	<1	<1	<1	<1	<1	<1
Western blot						
[dimers %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[aggregates %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[degradation products %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SDS-PAGE, silver stain						
[monomers %]	-	>>99%	>>99%	>>99%	>>99%	>>99%
[additional bands %]	-	n.d.	n.d.	n.d.	n.d.	n.d.
[degradation products %]	-	<<1%	<<1%	<<1%	<<1%	<<1%

n.d. = not detectable

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Claims

1. Lyophilized pharmaceutical preparation of G-CSF containing maltose, raffinose, sucrose, trehalose or amino sugar.

2. Lyophilized preparation as claimed in claim 1 additionally containing a physiologically tolerated amount of surfactant which is smaller than or at most the same as the amount of G-CSF protein used.

3. Lyophilized preparation as claimed in claim 2 containing 0.5 mg/ml, preferably 0.01 - 0.1 mg/ml surfactants.

4. Lyophilized preparation as claimed in one of the claims 1 to 3, which additionally contains a physiologically tolerated amount of amino acids.

5. Lyophilized preparation as claimed in claim 4 containing arginine and/or phenylalanine.

6. Lyophilized preparation as claimed in one of the claims 1 to 5 containing physiologically tolerated auxiliary substances selected from the group of antioxidants, complexing agents, buffers, acids, bases or isotonizing agents.

7. Lyophilized preparation as claimed in one of the claims 1 to 6 containing phosphate or acetate buffer.

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8. Lyophilized preparation as claimed in one of the claims 1 to 6 containing arginine phosphate, arginine chloride or arginine citrate buffer, preferably at a pH value of 7 - 8.

9. Lyophilized preparation as claimed in one of the claims 1 to 8, wherein it is essentially free from protein-like auxiliary substances or polymeric auxiliary substances.

10. Aqueous pharmaceutical preparation obtainable by redissolution of the lyophilisate as claimed in one of the claims 1 to 9.

11. Aqueous pharmaceutical preparation as claimed in claim 10, wherein the solution has a pH value of 6.5 - 8 or 3 - 5, preferably a pH value of 7.0 - 7.5.

12. Process for the production of a lyophilized pharmaceutical preparation as claimed in one of the claims 1 - 9, wherein an aqueous preparation is prepared which contains G-CSF as the active substance and maltose, raffinose, sucrose, trehalose or amino sugar as auxiliary substances as well as, if desired, further pharmaceutical auxiliary substances and the solution is subsequently lyophilized.

13. Use of maltose, raffinose, sucrose, trehalose or amino sugar for the production of a stable lyophilized pharmaceutical preparation containing G-CSF as the active substance.

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Abstract

STABLE LYOPHILIZED PHARMACEUTICAL PREPARATIONS OF G-CSF

The present invention concerns lyophilized pharmaceutical preparations of G-CSF that contain maltose, raffinose, sucrose, trehalose or amino sugar as stabilizing agents. In additon the invention concerns a process for the production of stabilized lyophilisates as well as the use of maltose, raffinose, sucrose, trehalose or amino sugar as stabilizing agents of pharmaceutical agents containing G-CSF.

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☑ Description : paged 1 to paged 38
☐ Claim(s) : paged ..... to paged .....
☐ Drawing(s) : paged ..... to paged .....
☐ Abstract : paged ..... to paged .....
DATE OF RECEIPT 8 Aug 1996

Boehringer Mannheim GmbH
3771/00/WO

Stable lyophilized pharmaceutical preparations of G-CSF

The present invention concerns lyophilized pharmaceutical preparations of G-CSF which contain maltose, raffinose, sucrose, trehalose or amino sugars as stabilizers. In addition the invention concerns a process for the production of these stabilized lyophilisates and the use of maltose, raffinose, sucrose, trehalose or amino sugars as stabilizers of pharmaceutical agents containing G-CSF.

Various pharmaceutical preparations which contain G-CSF (granulocyte colony stimulating factor) are already known from the state of the art.

A pharmaceutical agent containing G-CSF is described in DE 37 23 781 (GB 2,193,631) which contains at least one pharmaceutically acceptable surface-active agent, saccharide, protein or a high-molecular compound for stabilizing G-CSF. Preparations are proposed there which contain human serum albumin as a stabilizing agent. In particular preparations are stated to be advantageous which contain surface-active agents in parts by weight which correspond to 1 - 10000-fold the amount of G-CSF used.

Stabilized preparations of G-CSF are described in EP 0 373 679 which are characterized essentially by an acidic pH value of the solution, wherein the solutions should have as low a conductivity as possible. The

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solutions have a pH value of 3 - 3.7 in the case that the solutions contain further pharmaceutical auxiliary substances such as for example buffers or mannitol. If no buffer substances are present in the pharmaceutical formulation, pH ranges of 2.75 - 4 are described as being advantageous.

Furthermore, stabilized lyophilisates of human protein preparations are described in EP 0 306 824 in which stabilization is achieved by adding a mixture of urea, amino acids and detergent.

In the earlier PCT Patent Application PCT/EP92/01823 a process is described for the production of well-tolerated pharmaceutical agents containing G-CSF for infusion or injection purposes. The liquid forms of administration are characterized in particular by a low titration acidity and low buffer capacity. The pH values of the described infusion and injection solutions containing G-CSF are in an acidic range of about 3.8 - 4.5.

Processes for the production of liquid drug administration forms containing G-CSF which additionally contain preservatives are known from PCT/EP92/01822. The pH values of the aqueous pharmaceutical solutions are in the acidic range of 2.5 - 4.5. In this case the stabilization of G-CSF is essentially achieved by setting an acidic pH value which is favourable for G-CSF and by adding a mixture of various amino acids.

The previously known drug administration forms for G-CSF do, however, have some disadvantages. It was found that liquid G-CSF preparations can in some cases be sensitive

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towards freezing and thawing. The uncontrolled freezing and thawing of such preparations can lead to the formation of dimers, oligomers and aggregates; insoluble precipitates may also be formed. Such properties of pharmaceutical agents containing protein are questionable from a medical-pharmaceutical point of view since it is not possible to absolutely avoid an accidental freezing of the pharmaceutical solution and thus there is a risk of administering a qualitatively changed preparation.

A disadvantage of the preparations described in DE 37 23 781 is moreover that they contain pharmaceutical additives or auxiliary substances which, from a medical point of view, cannot simply be judged as harmless. Polymers and proteins have a certain potential risk with regard to their suitability as pharmaceutical additives due to their origin and their physicochemical properties. Proteins of human or animal origin as well as proteins obtained from cell cultures bear a potential residual risk of viral contaminations. Other protein-like impurities which are analytically difficult to detect can also cause immunological reactions in humans due to their antigenic properties. Moreover proteins of animal origin can generally trigger immunological reactions in humans due to their species-specific properties. Long-term reactions after a later re-administration of such proteins are also possible.

The addition of high-molecular compounds (polymers) may also be problematic. Polymers can accumulate in the body due to their large molecular mass and can thus remain in the body over a long period if no biodegradation occurs. This is a particular risk in the case of subcutaneous administration since removal and distribution via the

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blood stream is considerably slower compared to intravenous administration. Polymers may also have antigenic properties depending on their molecular mass. In addition the purity of polymers is difficult to guarantee because of the catalysts used for their production or the presence of monomers and other polymer fragments. The use of polymers in pharmaceutical forms of administration should thus be avoided if possible in particular in the case of forms of pharmaceutical agents that can be administered subcutaneously.

The amounts of surfactant described in DE 37 23 781 must also be regarded as problematic from a medical point of view. Here surfactant concentrations are described as being advantageous in which 1 to 10000 parts by weight of a surface-active agent are present in relation to the proportion by weight of G-CSF. If, on the other hand, one considers the preferred application concentrations of
G-CSF for clinical use of 0.05 - 1.5 mg/ml in the final pharmaceutical formulations, then this results in correspondingly high surfactant concentrations. These should be avoided from a medical point of view since they can cause local irritations.

In addition some of the known formulations have the disadvantage that, particularly in the case of subcutaneous administration, they lead to local intolerances in patients due to the low pH value used. The product obtained can cause pain and local tissue irritation in sensitive patients since the physiological pH range of 7.0 - 7.5 present in tissue is not adhered to.

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In addition it is known from the literature that especially non-glycosylated forms of G-CSF are particuarly unstable compared to glycosylated G-CSF which is obtained from CHO cells (J. Biol. Chem. 1990, 265, 11432). The stabilization of non-glycosylated forms of G-CSF proved to be particularly difficult and requires specially selected measures in order to formulate this molecule in a stable drug administration form.

The object of the present invention was to provide a drug administration form of G-CSF which enables a proper use of G-CSF as a pharmaceutical agent and which does not have the disadvantages of the previously known drug forms described above. The pharmaceutical preparation should be stable towards uncontrolled freezing and thawing processes as well as also being stable when stored for long periods as a lyophilisate, physiologically well-tolerated, simple to use and possible to dose precisely.

The examples described in DE 37 23 781 show that stable lyophilisates can be obtained when human serum albumin is used as an auxiliary agent. The addition of sugar alcohols alone leads to less stable formulations. Therefore with regard to improving the state of the art it is desirable to find formulations which do not contain human serum albumin (HSA) or other proteins or polymers but nevertheless have a good stability even at increased temperatures. The absence of human serum albumin and polymers reduces the medical risk of side-effects such as those described for example for HSA.

Surprisingly it was found that, within the sense of the

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present invention, it is possible to produce stable forms of pharmaceutical agents when maltose, raffinose, sucrose, trehalose or amino sugars are used as additives.

Solid preparations which contain maltose, raffinose, sucrose, trehalose or amino sugars as auxiliary agents can be frozen or even stored at increased temperatures (up to 40°C) with no significant loss of protein quality. The pharmaceutical quality of the active substance is not adversely affected by this. The preparations according to the invention are preferably put on the market as lyophilisates. The aqueous preparations prepared after redissolving are very well-tolerated and represent high quality preparations with regard to protein stability. In addition they have the advantage that the addition of maltose, raffinose, sucrose, trehalose or amino sugars as auxiliary agents enables solutions to be prepared with an advantageous pH value of 4 - 5 or 7 - 8 whereas the solutions known from the state of the art in general require solutions with a pH value of 2.5 - 3.5 for stabilizing the protein.

An additional advantage of the preparations according to the invention is that they are essentially free of protein-like or polymeric auxiliary substances the use of which may be problematic from a medical point of view. Due to the fact that liquid drug administration forms containing G-CSF obtained by dissolving lyophilisates can now be produced with a pH value of about 4 - 5 or 7 - 8, preferably with a pH value near to the pH value of blood (pH 7.2 - 7.4), they also have the advantage of being well tolerated and capable of administration substantially free of pain. This is particularly important for subcutaneous administration

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since in this case intolerances occur more easily than with intravenous administration. The preparations according to the invention can also be prepared in the clinically particularly preferred concentration ranges of 0.05 - 1.5 mg/ml so that it is possible to keep to injection volumes of $\leq$ 1.0 ml. Small injection volumes are particularly advantageous for subcutaneous administration since they cause only slight mechanical stimuli in the hypodermis.

A further advantage is that due to the selected auxiliary substances, the relatively high amounts of surfactant previously required in the liquid pharmaceutical preparations are no longer necessary. On the contrary low amounts of surfactant of 0.5 mg/ml or less, preferably of 0.01 - 0.1 mg/ml, are adequate to stabilize G-CSF. Surfactant concentrations (mg/ml) can be used advantageously which are smaller than or at most the same as the amount of G-CSF protein used per unit of volume (mg/ml). This is a particular advantage for those liquid drug forms which are intended for the subcutaneous administration of G-CSF. In addition the measures according to the invention lead to an adequate stabilization for pharmaceutical preparations of the labile non-glycosylated G-CSF molecules in particular.

The auxiliary agent maltose (malt sugar, maltobiose, 4-O-alpha-D-glucopyranosyl-D-glucose) is used in an amount of 0.01 - 10000-fold the amount of the active substance G-CSF. The same applies to the auxiliary substances raffinose, sucrose and trehalose. The concentration of these auxiliary substances in the liquid form of the pharmaceutical agent is 0.1 - 200 mg/ml, preferably 10 - 60 mg/ml. The stereoisomeric disaccharides cellobiose, gentiobiose or isomaltose can also be used instead of

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maltose. Amino sugars generally denote those monosaccharides which have an amino or an acylated amino group instead of a hydroxy group. Examples of this are glucosamine, galactosamine and neuraminic acid.

In a particular embodiment pharmaceutical preparations are provided which contain amino acids in addition to maltose, raffinose, sucrose or trehalose. Basic amino acids come into particular consideration as amino acids such as for example arginine, lysine, ornithine etc., acidic amino acids such as for example glutamic acid, aspartic acid etc. and also aromatic amino acids such as phenylalanine, tyrosine, tryptophan etc.

Amino acids are used in 0.01 - 10000-fold the amount of the active substance G-CSF. The concentration of these auxiliary substances in the liquid pharmaceutical preparation is 0.1 - 200 mg/ml, preferably 1 - 50 mg/ml.

In order to produce the lyophilisates, firstly the aqueous pharmaceutical solutions which contain the active substance and other common pharmaceutical auxiliary sustances are prepared. Amino acids such as e.g arginine, lysine, ornithine, phenylalanine or tyrosine come in particular into consideration as pharmaceutical auxiliary substances. In addition the aqueous preparation can contain common buffer substances such as e. g. acetic acid, hydrochloric acid, citric acid, lactic acid, tartaric acid, maleic acid and phosphoric acid or physiologically tolerated salts thereof. In the production of the auxiliary substance solution these buffer substances can either be present in the form of the corresponding free acids or in the form of the alkali, alkaline-earth or ammonium salts.

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The solution can in addition contain further common pharmaceutical auxiliary substances.

The sequence of addition of the various auxiliary substances or of the active substance is largely independent of the production process and is at the discretion of the person skilled in the art. The desired pH value of the solution is adjusted by adding bases such as alkali hydroxides, alkaline-earth hydroxides or ammonium hydroxide. Sodium hydroxide is preferably used for this. The adjustment of the desired pH value can in principle be achieved by adding basic solutions. In general salts of strong bases with weak acids come into consideration for this such as e.g. sodium acetate, sodium citrate, di-sodium or di-potassium hydrogen phosphate or sodium carbonate. If the pharmaceutical solution of auxiliary substance has a basic pH value, it is adjusted by titration with an acid until the desired pH range is reached. Physiologically tolerated inorganic or organic acids come into consideration as acids such as for example hydrochloric acid, phosphoric acid, acetic acid, citric acid or conventional solutions of substances which have an acidic pH value. In this respect preferred substances are salts of strong acids with weak bases such as e.g. sodium dihydrogen phosphate or potassium dihydrogen phosphate.

The concentrations of the buffer substances in the ready-to-administer liquid drug administration form are preferably about 2 - 80 mmol/l in each case. The total concentration of buffer substances should not exceed a value of 100 mmol/l. The concentration of buffer substances is preferably 5 - 40 mmol/l.

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The stabilization of G-CSF molecules by means of the said auxiliary substances relates in principle to all G-CSF molecules produced by recombinant processes and variants thereof. The term G-CSF or G-CSF variant according to the present invention includes all naturally occurring variants of G-CSF as well as G-CSF proteins modified by recombinant DNA technology that are derived therefrom, in particular fusion proteins which additionally contain other protein sequences in addition to the G-CSF part. In this regard a G-CSF mutein is particularly preferred with a N-terminal Met residue at position -1 which is suitable for expression in prokaryotic cells. A recombinant methionine-free G-CSF variant which can be produced according to PCT/EP91/00192 is equally suitable. The term "G-CSF variant" is understood to include those G-CSF molecules in which one or several amino acids may be deleted or replaced by other amino acids wherein the essential properties of G-CSF are substantially retained. Suitable G-CSF muteins are described for example in EP 0 456 200.

It is expedient to add auxiliary substances which act isotonically for the production of well-tolerated parenteral drug administration forms if isotonicity cannot be already achieved by the osmotic properties of the active substance and the auxiliary substances used for stabilization. For this purpose non-ionised, well-tolerated auxiliary substances are mainly used.

It is not advantageous to add salts to adjust the isotonicity, since high concentrations of salts or ions promote the formation of G-CSF aggregates. Therefore salts are advantageously added in small amounts.

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The pharmaceutical preparations can also contain further convential auxiliary substances or additives. Antioxidants such as for example glutathione, ascorbic acid or similar substances, chaotropic auxiliary substances such as for example urea and amino acids such as for example arginine, lysine, ornithine, glutamic acid and others can be added.

The invention is described in more detail in the following on the basis of representative examples of embodiments:

Examples 1 - 14 show in which manner lyophilisates according to the invention can be formulated, manufactured and examined in more detail with regard to the stability of the protein. The influence of the auxiliary substances added in addition to maltose, raffinose, sucrose or trehalose and of the pH value is elucidated.

Comparative experiments on lyophilisates produced on the basis of mannitol or glycine show that maltose, raffinose, sucrose or trehalose lyophilisates produce significantly better results than preparations prepared with other builders. Use of the lyophilisates described according to the invention and elucidated in the examples enables an optimal formulation to be produced for the described objective, which has a physiologically tolerated pH value, has a long-term storage stability enduring increased storage temperatures as well as mechanical stress without negative effects on the protein. The preparations are in particular not sensitive to freezing and it is possible to completely dispense with auxiliary substances that are regarded as

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being critical such as proteins or polymers. In addition they contain only relatively small amounts of physiologically well-tolerated surfactants.

Various sugars or sugar alcohols are examined in example 3 for their stabilizing effect in G-CSF lyophilisates. Maltose turns out to be advantageous compared to lactose and mannitol.

Lyophilisates containing maltose and further auxiliary substances are described in example 4. The results clearly demonstrate that the addition of surfactant does not substantially influence the stability of the preparation, but it prevents the adhesion of the protein to surfaces and thus prevents possible losses in content. The presence of surfactant in such formulations is thus not necessary for reasons of stabilization but rather to maintain the nominal dosage.

Various lyophilisate formulations containing maltose are compared in example 5 with two lyophilisates without maltose which are otherwise formulated identically. The data clearly show that the presence of maltose has an advantageous effect on the examined parameters with respect to stability of the preparation. The addition of further auxiliary substances such as ascorbic acid, glutathione or glutamic acid has no significant influence on the stability within the framework of the examined storage temperatures and storage periods. The preparations described in example 5 are distinguished in particular by the fact that they exhibit no changes in the examined quality criteria on long-term storage at an increased temperature.

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Furthermore it is apparent from the examples that a further buffer salt is not absolutely necessary in lyophilisates which contain maltose and arginine since the arginine buffer formed when the pH is adjusted by hydrochloric acid, phosphoric acid, citric acid or other acids has an adequate pH-stabilizing effect. Arginine buffer is extremely suitable for formulating stable preparations in the pH range under 5.0 and of 7.0 - 7.5 (see examples 11 and 12). Example 9 shows that redissolved lyophilisates with a pH of 7.4 and containing maltose and arginine buffer are stable for at least 24 hours.

G-CSF lyophilisates are described in example 6 which contain amino sugars (galactosamine, N-methyl-glucosamine). It can be seen that the combination of maltose and amino sugar results in more stable preparations than the combination of glycine with amino sugars. This demonstrates that maltose in combination with physiologically well-tolerated auxiliary substances yields considerably more stable and thus higher quality lyophilisates of G-CSF with regard to pharmaceutical quality than other builders and stabilizers proposed in the literature.

Example 7 shows that G-CSF is considerably more stable in lyophilisates containing maltose than in lyophilisates containing mannitol. This is demonstrated accordingly at relevant storage temperatures and for long storage periods.

Example 8 shows that lyophilisates containing maltose at various pH values and containing various auxiliary substance additives give advantageous results compared

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to lyophilisates with other builders and stabilizers (sugar alcohols, amino acids).

Example 10 demonstrates the stability of the lyophilisates according to the invention containing maltose, raffinose, sucrose or trehalose after 13 weeks storage at 40°C.

Example 11 shows that the lyophilisates according to the invention are stable even with higher G-CSF concentrations and the long-term stability of the formulations according to the invention even at increased temperatures is substantiated in example 12.

Example 1:

Test methods for the stability determination

The lyophilized preparations were stored in the dark at defined storage temperatures and subsequently examined with reversed phase HPLC (RP-HPLC), gel chromatography or size exclusion chromatography (SEC HPLC) and Western blot for protein purity as well as for the occurrence of aggregates and dimers. In addition the protein content was examined by OD 280 photometry, the biological activity by bioassay (NFS 60 cell test) and aggregation and precipitation by turbidity measurement. The methods used can be described as follows:

1.1 Reversed phase HPLC

The RP-HPLC was carried out using a Nucleosil C18 column (Knauer Company). The mobile phase consisted

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of 0.12 % (v/v) trifluoroacetic acid (TFA)/water (A) and 0.1 % (v/v) TFA/acetonitrile (B). The chromatography was carried out at a flow rate of 0.5 ml/min using a linear gradient of A to B.

The injection amount was 3 - 6 µg G-CSF depending on the formulation. It was evaluated at a wavelength of 214 nm by means of the peak area using an external standard.

1.2 Size exclusion chromatography (SEC)

A TSK G 2000 SW separation column (7.5 x 300 mm) was used for the SE chromatography. The separations were carried out isocratically at room temperature and a flow rate of 0.6 ml/min in a phosphate buffer (22.2 mM Na_2HPO_4; 107.7 mM KH_2PO_4; pH 6.2). The injected amount was 3 - 6 µg G-CSF. It was evaluated at a detection wavelength of 214 nm by means of the peak area using an external standard.

1.3 SDS page/Western blot

3 µg rhG-CSF is applied under non-reducing conditions to a 12 per cent polyacrylamide SDS gel and subjected to gel electrophoresis. Subsequently the G-CSF monomers, dimers or aggregates separated according to their molecular weight are transferred by electro-blotting onto nitrocellulose. The protein bands are identified by incubation with a specific polyclonal biotinylated anti-G-CSF antibody (PAB < GCSF > IgG) and detected by means of the phosphatase technique using streptavidin-alkaline phosphatase conjugate (SA-AP conjugate),

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5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). The percentage amounts of monomers, dimers and aggregates are determined by laser densitometric evaluation with the aid of a series of rhG-CSF standards.

1.4 NFS-60 bioassay (biological activity)

The in vitro determination of G-CSF activity is based on the measurement of cell counts in a cell culture of NFS-60 cells stimulated by G-CSF.

Under suitable conditions it is possible to correlate the dehydrogenase activity of the cells with the concentration of G-CSF in the medium. Suitable dilutions of the G-CSF buffer solution are prepared in order to obtain a readily measurable increase in the dehydrogenase activity.

The activity is then measured photometrically at 570 and 690 nm; the reduction of the tetrazolium salt MTT (yellow) to formazan (blue) is measured.

The in vitro activity of G-CSF is calculated by comparing the data for the sample with those for the standard according to the parallel line method. The evaluation is according to the requirements of Ph. Eur. (VIII, 13).

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1.5 Scattered light measurement, turbidity measurement

The measurement is carried out directly on the undiluted product solution in glass cuvettes (diameter 2 cm). The scattered light that is diffusely deflected by the liquid is measured at an angle of 90 degrees. It is measured in comparison to formazine standard suspensions according to DIN 38404C2, the values are stated in TU/F. The measurement is carried out on a suitable turbidity photometer, e.g. LTP 5 (Dr. Lange Company, Düsseldorf).

1.6 Photometry OD 280 (protein content)

The G-CSF UV spectrum has an absorbance maximum at 280 nm which is due to side chain chromophores such as tryptophan, tyrosine and phenylalanine residues. The measurement is carried out in comparison to placebo solutions by means of:

- UV spectrophotometer
  (e.g. Uvikon 810 P or 941, Kontron Instruments)

- Semi-micro quartz cuvettes, 500 µl, path length: 1 cm (e.g. Hellma, Suprasil, Cat. No. 104.002B-QS)

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Example 2:

Aqueous solutions of 0.1 mg/1 ml Poloxamer 118 and 50 mg/ml of the following sugars or sugar alcohols, mannitol (formulation 1), lactose (formulation 2) and maltose (formulation 3) were admixed with G-CSF at a concentration of 70 µg/ml. After filtration through a sterilized 0.2 µm membrane filter, the solutions were filled into sterile injection bottles made of glass of hydrolytic class I. After lyophilisation they were gassed with sterile nitrogen and the stoppers which were firstly put on loosely were pressed in under aseptic conditions to close the lyophilisates. The lyophilisates were flanged and stored in the dark for 6 and 13 weeks at different temperatures. Afterwards the stability of the preparation was examined with the methods described below.

Table 1a: Storage at 20°C

	6 weeks storage at 20°C			13 weeks storage at 20°C		
	I	II	III	I	II	III
Formul. 1 mannitol	>99%	91%	36%	86%	47%	27%
Formul. 2 lactose	>99%	>99%	18%	>99%	>99%	12%
Formul. 3 maltose	>99%	>99%	6%	>99%	>99%	7.8%

I Purity of unchanged protein in RP HPLC
II Purity of unchanged protein in SEC HPLC
III Dimers/aggregates in Western blot

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Table 1b: Storage at 40°C

	6 weeks storage at 40°C			13 weeks storage at 40°C		
	I	II	III	I	II	III
Formul. 1 mannitol	81%	70%	50%	69%	25%	70%
Formul. 2 lactose	>99%	>99%	37%	>99%	>99%	not detectable/ aggregated
Formul. 3 maltose	>99%	>99%	6.4%	>99%	>99%	12%

I Purity of unchanged protein in RP HPLC
II Purity of unchanged protein in SEC HPLC
III Dimers/aggregates in Western blot

Example 3:

Lyophilisates of G-CSF were prepared. For this the auxiliary substances stated in the following table were dissolved in water for injection purposes, subsequently G-CSF was added at a concentration of 70 µg/ml and if necessary the pH value was adjusted exactly with small amounts of the buffer system. Pluronic F 68 was used as a representative of an appropriate surfactant. Other surfactants behave similarly. After sterilization by filtration through a suitable 0.2 µm membrane filter, the solutions were filled into sterile injection bottles made of glass of hydrolytic class I and lyophilized according to conventional methods. After lyophilisation they were aerated with nitrogen and the injection bottles were closed under aseptic conditions with freeze-drying stoppers. The preparations were stored in flanged bottles in the dark at defined storage

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temperatures for 6 and 12 weeks and examined with the methods stated in example 1.

Table 2: Formulations of G-CSF at pH 3.6 containing maltose

	Formulation 4	Formulation 5
G-CSF	70 µg	70 µg
Maltose	35 mg	35 mg
L-Phenylalanine	10 mg	10 mg
Ascorbic acid	5 mg	5 mg
Glutathione	10 mg	10 mg
L-Glutamic acid	5 mg	5 mg
L-Arginine	10 mg	10 mg
Buffer (pH)	to pH 3.6	to pH 3.6
Pluronic F68	-	0.1 mg
Water for injection purposes	up to 1 ml	up to 1 ml

Table 3: Storage at 20°C

Fm.	Storage temp.	after 6 weeks aggr. in %	after 12 weeks aggr. in %	after 6 weeks SEC %G-CSF	after 6 weeks RP %G-CSF	after 12 weeks SEC %G-CSF	after 12 weeks RP %G-CSF
4	+20°C	0.0	2.6	61	63	64	69
5	+20°C	0.0	0.5	>99	>99	>99	>99

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Example 4:

G-CSF lyophilisates containing 500 µg/ml G-CSF (formulations 6 - 10) were prepared as follows. The auxiliary substances stated in the following table were dissolved in water for injection purposes, G-CSF was added and if necessary the pH value was adjusted with small amounts of hydrochloric acid or disodium hydrogen phosphate. In each case 1 ml of the solutions that were previously sterilized by filtration through a 0.2 µm membrane filter were filled into injection bottles made of glass of hydrolytic glass I and freeze-dried according to conventional methods. After the lyophilisation they were aerated with nitrogen and the lyophilisates were closed under aseptic conditions with freeze-drying stoppers. The flanged lyophilisates were stored in the dark at defined temperatures and examined using the methods stated in example 1.

Table 4: Compositions of formulations 6 - 10

	Formul.6	Formul.7	Formul.8	Formul.9	Formul.10
G-CSF	0.5 µg	0.5 µg	0.5 µg	0.5 µg	0.5 µg
Maltose	35 mg	35 mg	35 mg	-	-
L-Phenylalanine	10 mg	10 mg	10 mg	10 mg	10 mg
Ascorbic acid	5 mg	-	-	5 mg	-
Glutathione	10 mg	-	-	10 mg	-
L-Glutamic acid	5 mg	-	-	5 mg	-
L-Arginine	10 mg	10 mg	10 mg	10 mg	10 mg
Buffer (pH)	to 4.5	to 4.5	to 6.5	to 4.5	to 6.5
Pluronic F68	0.1 mg	0.1 mg	0.1 mg	0.1 mg	0.1 mg
Water for injection purposes	to 1 ml	to 1 ml	to 1 ml	to 1 ml	to 1 ml

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Table 5: Analytical results

Form	Storage temp.	Western blot 6 W %aggr.	Western blot 12 W %aggr.	after 6 weeks SEC %G-CSF	after 6 weeks SEC %aggr.	after 6 weeks RP %G-CSF	after 13 weeks SEC %G-CSF	after 13 weeks SEC %aggr.	after 13 weeks RP %G-CSF
6	+ 8°C	< 1	1.0	>99%	0.9	>99%	>99%	0.7	99%
	+ 40°C	< 1	1.7	>99%	0.6	>99%	>99%	0.6	98%
7	+ 8°C	< 1	1.1	>99%	1.6	>99%	>99%	1.1	99%
	+ 40°C	< 1	2.3	>99%	1.9	>99%	>99%	1.1	99%
8	+ 8°C	< 1	-				>98%	1.5	>99%
	+ 40°C	< 1	-				>98%	1.4	>99%
9	+ 8°C	3.8	0.3	95%	5.8	>99%	95%	1.5	98%
	+ 40°C	7.9	2.5	95%	6.4	93%	86%	0.8	94%
10	+ 8°C	-	5.2				96%	1.0	95%
	+ 40°C	-	10.3				89%	2.6	89%

Example 5:

The formulations stated in the following table (formulations 11 - 14) were prepared as follows: the auxiliary substances were dissolved in water for injection purposes, subsequently G-CSF was added at the stated concentration. If necessary the pH value was adjusted exactly with the aid of components of the phosphate buffer. The solutions were then filtered through a sterilized membrane filter with a pore size of 0.2 µm and filled under aseptic conditions into injection bottles of hydrolytic class I and lyophilized. After lyophilisation they were aerated with nitrogen, the lyophilisates were closed under aseptic conditions with freeze-drying rubber stoppers and flanged. The

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lyophilisates were stressed in the dark at defined storage temperatures. After 6 and 13 weeks they were examined with the methods stated in example 1.

Table 6: Lyophilisate preparations containing amino sugars

	Form. 11	Form. 12	Form. 13	Form. 14
G-CSF	0.5 µg	0.5 µg	0.5 µg	0.5 µg
Pluronic F68	0.1 mg	0.1 mg	0.1 mg	0.1 mg
N-methyl-glucosamine	-	10 mg	-	10 mg
Galactosamine	10 mg	-	10 mg	-
Glycine	-	-	35 mg	35 mg
Maltose	35 mg	35 mg	-	-
Phenylalanine	10 mg	-	-	-
Phosphate buffer	to pH 7.0	to pH 7.0	to pH 7.0	to pH 7.0
Water for injection purposes	up to 1.0 ml	up to 1.0 ml	up to 1.0 ml	up to 1.0 ml

The analytical data obtained after storage of the aforementioned preparations are summarized in the following results table.

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Table 7: Analytical results
DCP = decomposition products

Form.	Storage	6 Weeks West. blot % aggr.	12 Weeks West. blot % aggr.	12 Weeks RP-HPLC % G-CSF	12 Weeks % DCP in SEC HPLC
11	+ 8°C	3.8	2.9	>99	1.2
	+ 40°C	3.2	2.3	>99	1.8
12	+ 8°C	1.8	3.8	>99	1.4
	+ 40°C	1.7	4.5	>99	0.7
13	+ 8°C	1.1	1.4	>99	0.9
	+ 40°C	16.8	13.0	75	4.2
14	+ 8°C	1.6	12.4	97.5	1.2
	+ 40°C	7.7	26.3	84.5	3.5

Example 6:

Formulations 15 and 16 described in the following were prepared as follows: The stated auxiliary substances were dissolved in water for injection purposes, G-CSF was added in the stated concentration. The pH was adjusted if necessary using portions of buffer components. Afterwards the solution was filtered through a sterile membrane filter with a pore size of 0.2 µm and dispensed under aseptic conditions into sterile injection bottles made of glass of hydrolytic class I. Subsequently the injection preparations were freeze-dried, then aerated with nitrogen and the injection bottles were closed under aseptic conditions with freeze-drying stoppers and subsequently flanged. The formulations were stored in the dark at defined temperatures and examined for the parameters mentioned below. The test methods stated in example 1 were used for this.

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Formulation 15

G-CSF	**0.25**
Polysorbate 80	0.05 mg
Phenylalanine	5 mg
Maltose	17.5 mg
L-arginine	5 mg
Buffer	to pH 4.5
Water for injection purposes	up to 0.5 ml

Formulation 16

G-CSF	**0.3 mg**
Polysorbate 80	0.1 mg
Mannitol	50 mg
Buffer	to pH 4.5
Water for injection purposes	up to 1.0 ml

Table 8: Examination results after storage of formulations 15 and 16 for 3 and 6 months

	Formulation 15 storage period 3 months		storage period 6 months		Formulation 16 storage period 3 months		storage period 6 months	
	4-8°C	23°C	4-8°C	23°C	4-8°C	23°C	4-8°C	23°C
West.blot (dimers)	2.2	< 1%	1.3%	0.7	< 1%	12%	4.1%	17
SEC-HPLC (dimers)	< 1%	<1%	< 1%	< 1%	< 1%	2%	< 1%	3
RP-HPLC (G-CSF peak)	>99%	>99%	>98%	>98%	>99%	98.2%	>98%	>98

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Example 7:

The formulations described in table 9 were prepared as follows:

The stated auxiliary substances were dissolved in water for injection purposes, G-CSF was added in the stated concentration, afterwards the pH was adjusted if necessary with small portions of buffer components. The pharmaceutical solution was then filtered through a sterilized membrane filter with a pore size of 0.2 µm and subsequently dispensed into sterile injection bottles made of glass of hydrolytic class I under aseptic conditions and lyophilized.

After lyophilisation they were aerated with nitrogen and the bottles were closed under aseptic conditions with freeze-drying stoppers. The bottles were flanged and stored in the dark under defined temperature conditions. After the respective storage periods, analytical examinations were carried out using the methods described in example 1 (cf. table 10).

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Table 9: G-CSF lyophilisates containing mannitol compared to other builders

	Form. 17	Form. 18	Form. 19	Form. 20	Form. 21	Form. 22	Form. 23	Form. 24
G-CSF	350 µg	350 µg	175 µg	175 µg	175 µg	175 µg	175 µg	175 µg
Maltose			17.5 mg	17.5 mg	17.5 mg	-	-	-
Glycine						4 mg	10 mg	8.9 mg
Arginine			5 mg	-	5 mg	5 mg	-	
Phenylalanine			5 mg	5 mg	5 mg	5 mg	-	2.5 mg
Mannitol	50 mg	50 mg				-	-	
Tween 80	0.1 mg	0.1 mg	0.05 mg	0.05 mg	0.05 mg	0.05 mg	0.05 mg	0.05 mg
pH	4.5	7.2	4.5	7.2	7.2	7.2	7.2	7.2
(buffer)	HCl	phosphate	HCl	HCl	HCl	HCl	phosphate	phosphate
Water for injection purposes	up to 1.0 ml	up to 1.0 ml	up to 0.5 ml	up to 0.5 ml	up to 0.5 ml	up to 0.5 ml	up to 0.5 ml	up to 0.5 ml

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In Table 10 the analytical data are summarized for the stated formulations:

Formulation	Storage	4 weeks RP-HPLC % G-CSF	4 weeks SEC-HPLC % G-CSF	4 weeks Western blot
17	8°C	> 99	> 99	3.6 % dimers
	30°C	94	92	9.6 % dimers
	40°C			14.4 % dimers
18	8°C	69	60	aggregates
	30°C	44	36	aggregates
	40°C	13	12	aggregates
19	8°C	> 99	> 99	1.0 % dimers
	30°C	> 99	95.5	0.5 % dimers
	40°C	> 99	97.5	0.5 % dimers
20	8°C	> 99	> 99	1.6 % dimers
	30°C	> 99	> 99	1,4 % dimers
	40°C	> 99	> 99	2.3 % dimers
21	8°C	> 99	> 99	1.5 % dimers
	30°C	> 99	97.5	2.1 % dimers
	40°C	> 99	97	2.0 % dimers
22	8°C	> 99	> 99	2.8% di/aggr.
	30°C	96	96	3.0% di/aggr.
	40°C			12 % di/aggr.
23	8°C	> 99	> 99	6.8 % dimers
	30°C	91.5	92	aggregates
	40°C	79	74	aggregates
24	8°C	> 99	> 99	10.8 % dimers
	30°C	88	85	aggregates
	40°C	67	60	aggregates

Example 8:

Standing time of redissolved lyophilisates according to the invention having a pH of 7.4

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The following composition was prepared:

mg/ml	Formulation 25
G-CSF	0.35
Polysorbate 80	0.1
Maltose	50
Arginine	10
Phenylalanine	10
Hydrochloric acid	to pH 7.4

The stated auxiliary substances were dissolved in 1 ml water for injection purposes, G-CSF was added and the pH value was adjusted to pH 7.4. The solution was sterilized by filtration through a sterilized membrane filter with a pore size of 0.2 µm and afterwards dispensed into injection bottles of hydrolytic class I.

After putting on suitable freeze-drying stoppers, the preparation was freeze-dried at a main drying temperature of -25°C and a subsequent drying temperature of +8°C until the residual moisture reached < 5 %. The dried lyophilisates were aerated with nitrogen and closed.

After 6 months storage at 4 - 8°C the lyophilisates were dissolved in 1 ml water for injection purposes and allowed to stand for 24 hours at room temperature.

After this standing time there was no change using the examination methods described in example 1 with regard to biological activity (NFS-60 test), protein content (photometry OD 280) and purity (Western blot), purity (SEC HPLC), purity (SDS page) and purity (RP HPLC) compared to samples examined directly after dissolution. Turbidity measurements - even under

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mechanical stress - resulted in very low turbidity values.

This clearly indicates that redissolved lyophilisates of the formulation according to the invention containing maltose and arginine buffer at pH 7.4 have an adequate standing time for clinical application.

Example 9:

Stability of lyophilisates according to the invention containing maltose, raffinose, sucrose or trehalose after 13 weeks storage at 40°C

Three lyophilisates corresponding to example 8, formulation 25, were prepared that contained 50 mg/ml maltose or the same amount by weight of a) raffinose or b) sucrose or c) trehalose.

All lyophilisates were stored for 13 weeks at temperatures of 5°C, 25°C, 30°C and 40°C, afterwards they were dissolved and examined visually and using the examination methods SEC HPLC, RP HPLC, Western blot and SDS page described in example 1.

In all cases clear colourless solutions resulted. In SEC HPLC the product peaks had a size of > 98 % and the dimers/aggregates had a size of < 1%. In RP HPLC the product peak reached 100 %, secondary peaks could not be detected, the main peak corresponded to the working standard. In SDS-page no degradation products, dimers or aggregates could be detected.

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Table: after 13 weeks storage period

Temp.	SEC HPLC % G-CSF	RP HPLC % G-CSF	Western blot % aggregates	SDS-Page % minor bands
5°C	>98%	100%	n.d.	<1
20°C	>98%	100%	n.d.	<1
30°C	>98%	100%	n.d.	<1
40°C	>98%	100%	n.d.	<1

Example 10:

Stability of maltose lyophilisates according to the invention containing arginine phosphate and arginine chloride buffers at pH 4.5 and pH 7.2 after 13 weeks storage at 30°C

The following formulations were prepared according to the production process described in example 8, which only differed in their buffers and pH values:

	Formulation 26	Formulation 27	Formulation 28	Formulation 29
G-CSF	0.35 mg	0.35 mg	0.35 mg	0.35 mg
Polysorbate 80	0.1 mg	0.1 mg	0.1 mg	0.1 mg
Phenylalanine	10 mg	10 mg	10 mg	10 mg
Arginine	10 mg	10 mg	10 mg	10 mg
Maltose	47.5 mg	47.5 mg	47.5 mg	47.5 mg
Phosphoric acid	to pH 4.5	to pH 7.2		
Hydrochloric acid			to pH 4.5	to pH 7.2

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The preparations were stored at temperatures of 4 to 8°C, 20 - 25°C and 30°C, dissolved after 13 weeks in 1 ml water for injection purposes and examined using the examination methods described in example 1 i.e. RP HPLC, SEC HPLC (purity) and Western blot (degradation, dimerization and aggregate formation). The results are shown in table 11 and show that the lyophilisates at pH 4.5 and 7.2 according to the invention are stable after 13 weeks storage at 30°C.

Table 11: Results after 13 weeks at 30°C

	RP-HPLC % minor peaks	SEC-HPLC	Western blot degrad.	dimers	aggregate
Formulation 26	<1	<1	n.d.	n.d.	n.d.
Formulation 27	<1	<1	n.d.	n.d.	n.d.
Formulation 28	<1	<1	n.d.	<1%	n.d.
Formulation 29	<1	<1	n.d.	<1%	n.d.

n.d. = not detectable

Example 11:

Stability of maltose lyophilisates according to the invention containing arginine phosphate and arginine chloride buffer at pH 7.4 after 4 and 13 weeks storage at 40°C

Formulations were prepared in the same manner as described in example 8 and their pH value was adjusted to 7.4 in one case with hydrochloric acid and once with

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phosphoric acid.

	Formulation 30	Formulation 31
G-CSF	0.35 mg	0.35 mg
Polysorbate 80	0.1 mg	0.1 mg
Phenylalanine	10 mg	10 mg
Arginine	10 mg	10 mg
Maltose	47.5 mg	47.5 mg
Phosphoric acid	pH 7.4	
Hydrochloric acid		pH 7.4

These two preparations were stored for 4 and 13 weeks at temperatures of 4 - 8°C and 40°C. The examination results (Western blot, SDS page) after 13 weeks storage are shown in the following table 12. The results after 4 weeks storage are identical.

The results show that the maltose lyophilisates at pH 7.4 according to the invention are stable after 13 weeks storage at 40°C.

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Table 12: Results after 13 weeks at 40°C

	Storage temp.	Western blot, non-reduc. aggr. <1%	dimers <2%	SDS-Page, reduc. product band	degr. prod.	other bands	Residual moisture %
Form. 30	5°C	n.d.	n.d.	<98%	<1%	n.d.	1.4
	40°C	n.d.	n.d.	<98%	<1%	n.d.	2.0
Form. 31	5°C	n.d.	n.d.	<98%	<1%	n.d.	1.9
	40°C	n.d.	n.d.	<98%	<1%	n.d.	2.1

Example 12:

Stability of lyophilisates according to the invention having a pH of 7.4 and G-CSF concentrations of 0.5 and 1.0 mg/ml after 13 weeks storage at 40°C

The following formulations were prepared according to the production process described in example 8 which differ in their content of G-CSF:

mg/ml	Formulation 32	Formulation 33
G-CSF	0.5 mg	1.0 mg
Polysorbate 80	0.1 mg	0.1 mg
Phenylalanine	10 mg	10 mg
Arginine	10 mg	10 mg
Maltose	47.5 mg	47.5 mg
Phosphoric acid	pH 7.4	pH 7.4

The formulations were stored for 4 weeks and 13 weeks at -20°C, 4 - 8°C, 20°C - 25°C, 30°C and 40°C, afterwards dissolved in 1 ml water for injection purposes and

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examined using the examination methods SEC HPLC, RP HPLC, Western blot and SDS-page described in example 1 (examination results see table 13).

The results show that the lyophilisates of the formulations according to the invention are stable even at a higher protein concentration of up to 1 mg/ml after 13 weeks storage at 40°C.

Example 14:

Long-term stability during 9 months

Lyophilisates were prepared using formulation 31 of example 11 and the preparations were stored for 9 months at temperatures of -20°C, 5°C, 25°C, 30°C and 40°C and examined after 3, 6 and 9 months using all examination methods described in example 1.

No change could be detected in any of the examined parameters during the storage period. At the end of the storage periods and at all temperatures the preparation proved to be fully biologically active, it had the complete protein content and showed bands or peaks in all determinations of purity which were well below 1 % of the intact G-CSF molecule.

The results show that lyophilisates according to the invention are stable even when stored for long periods at higher temperatures and thus greatly exceed the stabilites described in the state of the art.

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Table 14: Storage at 30°C

	3 months	6 months	9 months
NFS 60 test 80 - 125%	corresponds	corresponds	corresponds
OD 280	358 mg	360 mg	352 mg
SDS page minor bands	<1%	<1%	<1%
Western blot % aggregates %dimers	n.d. <1%	n.d. <1%	n.d. <1%

	3 months	6 months	9 months
RP HPLC Product peak	>99%	>99%	>99%
SEC HPLC Product peak Minor peaks	>98% n.d.	>98% n.d.	>98% n.d.
Turbidity measurement TU/F	0.5	0.5	0.5

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Table 13:

Formulation 32

Test parameter	0 weeks [refrigerator]	13 weeks [-20°C]	13 weeks [refrigerator]	13 weeks [RT]	13 weeks [30°C]	13 weeks [40°C]
Visual examination						
-appearance, lyophilisate	-	white	white	white	white	white
-clarity, (solution)	-	clear	clear	clear	clear	clear
SEC-HPLC						
[purity %]	>98	>98	>98	>98	>98	>98
[dimers/aggregates %]	<1	<1	<1	<1	<1	<1
RP-HPLC						
[purity %]	>98	>98	>98	>98	>98	>98
[sum of minor peaks %]	<1	<1	<1	<1	<1	<1
Western blot						
[dimers %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[aggregates %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[degradation products %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SDS-PAGE, silver stain						
[monomers %]	-	>>99%	>>99%	>>99%	>>99%	>>99%
[additional bands %]	-	n.d.	n.d.	n.d.	n.d.	n.d.
[degradation products %]	-	<<1%	<<1%	<<1%	<<1%	<<1%

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Formulation 33

Test parameter	0 weeks [refrigerator]	13 weeks [-20°C]	13 weeks [refrigerator]	13 weeks [RT]	13 weeks [30°C]	13 weeks [40°C]
Visual examination						
-appearance, lyophilisate	-	white	white	white	white	white
-clarity, (solution)	-	clear	clear	clear	clear	clear
SEC-HPLC						
[purity %]	>98	>98	>98	>98	>98	>98
[dimers/aggregates %]	<1	<1	<1	<1	<1	<1
RP-HPLC						
[purity %]	>98	>98	>98	>98	>98	>98
[sum of minor peaks %]	<1	<1	<1	<1	<1	<1
Western blot						
[dimers %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[aggregates %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[degradation products %]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SDS-PAGE, silver stain						
[monomers %]	-	>>99%	>>99%	>>99%	>>99%	>>99%
[additional bands %]	-	n.d.	n.d.	n.d.	n.d.	n.d.
[degradation products %]	-	<<1%	<<1%	<<1%	<<1%	<<1%

n.d. = not detectable

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SET #3

REPLACEMENT SHEETS SG
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☒ Claim(s) : paged 39 to paged 41
☐ Drawing(s) : paged ..... to paged .....
☐ Abstract : paged ..... to paged .....
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CLAIMS

1. Process for the production of lyophilized pharmaceutical preparations of G-CSF which are stable on storage, wherein an aqueous preparation of G-CSF and if desired further pharmaceutical auxiliary substances is prepared and subsequently the solution is lyophilized wherein the additives maltose, cellobiose, gentiobiose, isomaltose, raffinose, trehalose or amino sugar are added in the production of the aqueous solution in order to avoid loss of quality of G-CSF during uncontrolled freezing and thawing processes or during storage at increased temperatures.

2. Process for the production of lyophilized pharmaceutical preparations of G-CSF which are stable on storage, wherein an aqueous preparation of G-CSF and if desired further pharmaceutical auxiliary substances is prepared and subsequently the solution is lyophilized wherein the additives (1) maltose, raffinose, sucrose or trehalose and (2) a basic acidic or aromatic amino acid are added in the production of the aqueous solution in order to avoid the occurrence of aggregates or dimers of G-CSF after redissolution of the lyophilisate.

3. Process as claimed in claim 1, wherein maltose, raffinose, trehalose or amino sugar are used as additives.

4. Process as claimed in claim 3, wherein maltose, raffinose, glucosamine, galactosamine or neuraminic acid are used as additives.

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5. Process as claimed in one of the claims 1 - 4, wherein a physiologically tolerated amount of surfactants is added which is smaller than or at most the same as the amount of G-CSF protein used.

6. Process as claimed in claim 5, wherein surfactants are added in an amount of 0.5 mg/ml, preferably of 0.01 - 0.1 mg/ml.

7. Process as claimed in one of the claims 1 - 6, wherein a physiologically tolerated amount of amino acids is added.

8. Process as claimed in claim 7, wherein arginine and/or phenylalanine is added.

9. Process as claimed in one of the claims 1 - 8, wherein antioxidants, complexing agents, buffers, acids, bases or isotonizing agents are added as the physiologically tolerated auxiliary substances.

10. Process as claimed in one of the claims 1 - 9, wherein phosphate buffer or acetate buffer is added as the physiologically tolerated auxiliary substance.

11. Process as claimed in one of the claims 1 - 10, wherein arginine phosphate buffer, arginine chloride buffer or arginine citrate buffer preferably with a pH value of 7 - 8 is added as a physiologically tolerated auxiliary substance.

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12. Use of maltose, cellobiose, gentiobiose, isomaltose, raffinose, sucrose, trehalose, glucosamine, galactosamine or neuraminic acid to avoid loss of G-CSF quality during freezing or during storage at increased temperatures of pharmaceutical preparations containing G-CSF.

13. Lyophilized pharmaceutical preparation of G-CSF containing maltose, cellobiose, gentiobiose, isomaltose, raffinose, glucosamine, galactosamine or neuraminic acid.

14. Lyophilized pharmaceutical preparation of G-CSF containing (1) maltose, raffinose, sucrose or trehalose and (2) a basic, acidic or aromatic amino acid.

15. Lyophilized pharmaceutical preparation of G-CSF as claimed in claim 13 or 14 that is essentially free of protein-like or polymeric auxiliary substances.

16. Aqueous pharmaceutical preparation of G-CSF obtainable by redissolution of the lyophilisate as claimed in one of the claims 13 - 15.

17. Aqueous pharmaceutical preparation as claimed in claim 16, wherein the solution has a pH value of 6.5 - 8 or 3 - 5, preferably a pH value of 7.0 - 7.5.

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