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Stabilization of a recombinant human epidermal growth factor parenteral formulation through freeze-drying

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ABSTRACT

Development studies were performed to design a pharmaceutical composition that allows the stabilization of a parenteral rhEGF formulation in a lyophilized dosage form. Unannealed and annealed drying protocols were tested for excipients screening. Freeze-dry microscopy was used as criterion for excipients and formulation selection; as well as to define freeze-drying parameters. Excipients screening were evaluated through their effect on freeze-drying recovery and dried product stability at 50 °C by using a comprehensive set of analytical techniques assessing the chemical stability, protein conformation and bioactivity. The highest stability of rhEGF during freeze-drying was achieved by the addition of sucrose or trehalose. After storing the dried product at 50 °C, the highest stability was achieved by the addition of dextran, sucrose, trehalose or raffinose. The selected formulation mixture of sucrose and dextran could prevent protein degradation during the freeze-drying and delivery processes. The degradation rate assessed by RP-HPLC could decrease 100 times at 37 °C and 70 times at 50 °C in dried with respect to aqueous formulation. These results indicate that the freeze-dried formulation represents an appropriate technical solution for stabilizing rhEGF.

1. Introduction

About 347 million people are currently affected by diabetes mellitus (DM) [1]. World Health Organization (WHO) estimated that diabetes will be the 7th highest cause of death in 2030 [2]. Diabetic patients have a reduced capacity to carry out tissue repair processes, and are more susceptible to chronic wounds, such as leg ulcers and diabetic foot ulcers (DFU), that frequently cause hospitalizations, amputation and morbidity [3]. An estimated 15% of patients with DM develop foot ulcers. Ten-year mortality rates are increased to 50% in patients with DFU compared to patients without ulceration [4]. A wide range of medical interventions are used for DFU management and new therapies are emerging to promote wound healing, including topically applied growth factors [5], skin substitutes [6,7], and others that have shown efficacy in relatively small, pure neuropathic, non-complicated ulcers [8]. Amputation is still a foreseeable outcome in cases with large, advanced DFU, even more if ischemia is present.

The epidermal growth factor (EGF) induces mitogenic, motogenic, and cyto-protective actions that are instrumental for the healing process [9]. The availability of the growth factor at the deeper layers of the wound is an important issue to obtain an adequate therapeutic effect. This can be a limitation for topical formulations because the active agent bioavailability is affected by necrotic tissue, sepsis, inflammation and wound proteases [10]. Significantly, the addition of metalloproteinase inhibitors can revert the substantial degradation of exogenous EGF and its receptor in chronic ulcers, implying that EGF is susceptible to the proteolytic environment of such wounds [11]. The local infiltration of EGF into the wound base and edges possibly reduces its degradation following topical application and contact with wound

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exudates. Recently, a new first-in-class treatment based on local (intralesional) instillation of recombinant human EGF (rhEGF) to heal advanced DFU has been developed. The results of this intervention can be considered clinically relevant because they offer an alternative treatment for advanced ulcers (Wagner's grade 3 or 4, mostly above 20 cm^2 wide, including ischemic) at higher risk of amputation [12].

To use the rhEGF as local infiltrations drug for DFU treatment, it is necessary to develop a safe and effective formulation by stabilizing the protein during production and storage. EGF is a small protein, molecular weight 6230 g/mol, containing three internal disulfide bridges. Previous studies have identified the Asn-1 deamidation, the conversion of Asp-11 to a stable Asp-succinimide and Met-21 oxidation as critical factors that hindered rhEGF stabilization in a liquid formulation [13]. The modification of both Asn and Asp residues (Asx) is one of the major chemical degradative pathways for proteins during manufacturing and storage. Although the reactivity of Asx residues is highly reduced in proteins formulated as lyophilized powders in some lyophilized formulations, it could still be significant enough to compromise protein stability [14]. Conceivably, shelf life of rhEGF in dried state could be increased due to absence of water and dissolved oxygen; thereby circumventing protein hydrolysis and oxidation.

Since rhEGF is very labile in aqueous systems, it is highly relevant to develop and characterize a successful lyophilized dosage form [13]. Accordingly, in this work, a screening study was performed to identify the excipients which mitigate the protein degradation during freeze-drying and storage at high temperature. Two drying protocols, unannealed and annealed, were tested for initial screening. Freeze-drying microscopy was used as criterion for excipients and formulation selection; as well as to define freeze-drying parameters. The effects of the sucrose to dextran ratio on rhEGF stability during the freeze-drying process, and storage of the dried formulation at high temperature, were also determined. In addition, protein stability was monitored using a comprehensive set of analytical techniques assessing the formation of aggregates, protein conformation and biological activity. The properties of freeze-dried cake, namely the reconstitution performance and the residual moisture content, and the stability of the rhEGF formulation at high temperatures in liquid and dried states were analyzed.

2. Materials and methods

2.1. Materials

A mixture of rhEGF1-51 and rhEGF1-52 expressed in *Saccharomyces cerevisiae* was supplied as concentrated bulk solution (CIGB, Havana, Cuba). Chemicals were of analytical grade and the excipients met the European Pharmacopoeia standards (EP). Dextran with molecular weights of 37,000–43,000, indicated as dextran, was used (AppliChem GmbH, Darmstadt, Germany). Neutral clear borosilicate glass type I hydrolytic quality vials (Nuova OMPI, Piombino Dese, Italy), bromobutyl type gray siliconized freeze-drying rubber stoppers and flip-off aluminum seals covered with polypropylene plastic cap (Helvoet Pharma, Alken, Belgium) were used. Water for injection (WFI) and normal saline solution (9.0 g/L NaCl; NSS) (Quimefa, Havana, Cuba), as well as disposable 27-gauge × 0.5-inch needle (VWR Scientific, San Francisco, CA, USA) coupled to a 5-mL silicone oil free disposable syringe (Fisher Scientific, Pittsburgh, PA, USA) were used in the injection simulation studies.

Table 2
Effect of dextran/sucrose ratio on T_{oc} and lyophilized cake attributes of rhEGF formulations.

Weight fraction of dextran[a] (w/w)	T_{oc} (°C)	Appearance of cake	Moisture content (%, w/w)	Reconstitution time (s)	Absorbance 350 nm
0	−35.3 ± 0.4	Partial collapse	3.3 ± 0.3	10 ± 2	0.005 ± 0.002
0.25	−30.8 ± 0.5	Noncollapsed	3.1 ± 0.3	13 ± 3	0.005 ± 0.002
0.5	−26.0 ± 1.4	Noncollapsed	3.0 ± 0.2	14 ± 2	0.006 ± 0.001
0.75	−19.6 ± 0.9	Noncollapsed	3.6 ± 0.3	16 ± 2	0.007 ± 0.002
1.0	−14.1 ± 1.2	Noncollapsed	4.2 ± 0.3	18 ± 3	0.008 ± 0.003

[a] Weight fraction of dextran = (weight of dextran)/(weight of dextran + weight of sucrose).

2.2. Preparation of rhEGF formulations

Phosphate buffer was chosen since it stabilizes rhEGF in the presence of many excipients during the freeze and thawing process [13]. Furthermore, sodium phosphate buffer at 10 mM was selected to minimize pH shift during freezing and avoid an unnecessary decrease in maximal freeze concentrate temperature, Tg', of protein formulation [15]. For the current study, rhEGF excipients previously shown to be incompatible, after solution stressed at high temperature and multiple freezes and thawing cycles were discarded [13]. rhEGF at 75 μg/mL formulations were prepared with 2% w/v excipients or excipients mixtures in 10 mM phosphate buffer, pH 7.0. The solutions were filtered using 0.22 μm filters (Millipore, Bedford, Massachusetts, USA) prior to freeze-drying. The individual excipients tested are shown in Table 1, and the different sucrose-dextran mixtures tested in Table 2 as weight fraction of dextran (w/w). As a negative control, a rhEGF solution of 75 μg/mL (without any excipients) was freeze-dried at the same settings as the formulations.

Table 1
List of lyoprotectants used in this study with corresponding onset of collapse temperature (T_{oc}) and lyophilized cake attributes.

Excipient	T_{oc} (°C)	Cycle	Appearance	Moisture content (%, w/w)	Reconstitution time (s)	Absorbance 350 nm
Buffer (sodium phosphate)	−49.5 ± 0.5	Unannealed	White, shrunken	3.7 ± 1.4	6 ± 1	0.006 ± 0.001
Sucrose	−35.3 ± 0.4	Unannealed	White, slightly shrunken	3.1 ± 0.3	9 ± 2	0.005 ± 0.003
Trehalose	−32.4 ± 1.2	Unannealed	White, slightly shrunken	3.4 ± 0.6	11 ± 3	0.004 ± 0.004
Raffinose	−27.7 ± 0.9	Unannealed	White, non-shrunken	3.2 ± 0.4	13 ± 3	0.004 ± 0.002
Dextran 40	−14.1 ± 1.2	Unannealed	White, non-shrunken	3.9 ± 0.5	16 ± 2	0.009 ± 0.004
Sorbitol	−45.9 ± 1.7	Unannealed	White, shrunken	3.7 ± 0.5	29 ± 2	0.003 ± 0.002
Mannitol	−29.6 ± 0.8	Unannealed	White, non-shrunken	3.1 ± 0.3	9 ± 1	0.003 ± 0.001
		Annealed	White, non-shrunken	2.6 ± 0.3	10 ± 3	0.003 ± 0.001
Glycine	−9.7 ± 0.5	Unannealed	White, non-shrunken	2.8 ± 0.5	11 ± 1	0.005 ± 0.004
		Annealed	White, non-shrunken	2.3 ± 0.3	16 ± 2	0.004 ± 0.002

Sugar and polymer: sucrose, trehalose, raffinose and dextran-40.
Polyol: mannitol, sorbitol.
Amino acid: glycine.

2.3. Freeze-drying

Lyophilization vials, 2-mL type 1 glass tubing vials, were filled with 0.5 mL of formulations and partially stoppered with 13 mm lyophilization stoppers. The formulation of rhEGF in different excipients would require different freeze-drying conditions, thus two protocols were tested: the unannealed one which has been commonly used for amorphous carbohydrate (*e.g.*, sucrose, trehalose or dextran) and the annealed one that also included an annealing step as required to guarantee maximum crystallization (*e.g.*, for mannitol or glycine) [15].

Freeze-drying cycles were performed in an Edwards freeze dryer (Sussex, England) with a condensing capacity of 8 kg/h. The protocol applied for unannealed lyophilizates included a frozen hold step at −45 °C for 5 h. Primary drying was carried out at a −30 °C shelf temperature and chamber pressure of 120 μbar for 8 h, regulated by nitrogen injection. Secondary drying was carried out at a shelf temperature of 25 °C and chamber pressure of 75 μbar for 6 h. In the annealed protocol, after 2 h frozen at −45 °C, an annealing step for 2 h at −15 °C was applied. The frozen step finished after 5 additional *h* at −45 °C. Primary and secondary drying were the same as for the unannealed protocol. After finishing the cycle steps, the vials were stoppered inside the drying chamber, and then removed and crimped for characterization and stability studies.

2.4. Freeze-dry microscopy

Liquid samples were tested using a freeze-drying microscope (Biopharma Technology Ltda, Surrey, UK). This consisted of a small freeze-drying chamber connected to a temperature and pressure controlled stage; a vacuum pump and a microscope (Olympus BX-51, Hertfordshire, UK) equipped with a 10 × condenser extension lens (Linkam, Surrey, UK). Images were recorded using a monochrome video camera attachment (Imasys, Suresnes, France). A 2 μL liquid sample of the rhEGF formulations were placed between two cover slips on the thermal conductor and sealed within the stage. The samples were cooled at −50 °C and at a rate of 5 °C/min; a vacuum was then initiated within the stage and maintained about 80 μbar, and samples then heated to 25 °C at 1 °C/min. During the experiment, the pressure was monitored using a calibrated Pirani gauge. Freeze-dry microscopy experiments were rum in triplicate. The temperature, at which the initial structure change was detected, is referred to as the onset collapse temperature (T_{oc}), and the temperature, at which the structural change appeared complete throughout the product is referred to as the complete collapse temperature (T_{cc}).

2.5. Effect of freeze-drying and delivery processes on rhEGF stability

The freeze-drying process was evaluated comparing the protein characteristics (see below) on samples after rehydrating the cakes to their original volume (reconstituted-Lyo) to the one before the process (pre-Lyo). A simulated injection process was carried out to evaluate the overall effect of this procedure on the formulation stability, by comparing the protein characteristics on the sample after passing through needle and syringe (after-NS) with the reconstituted-Lyo sample one. To gain knowledge about the influence of reconstitution solvent and delivery process, lyophilized solid samples were reconstituted with WFI and NSS.

2.6. Lyophilizate characteristics

2.6.1. Water content

Water content was determined using a Karl Fischer coulometric titrimeter model TIM550 (Radiometer Analytical, Lyon, France). The residual moisture content was calculated based on the amount of water in the vial and cake weight.

2.6.2. Appearance and reconstitution

The lyophilized formulations were visually inspected for cake appearance. The reconstitution time of the lyophilized cakes was determined by adding their original volume of sterile water to vials and recording the time to obtain a completely clear solution. Samples were visually inspected post reconstitution for particulate matter, color and clarity. All experiments were carried out in a biosafety cabinet using sterile pipettes.

2.6.3. Differential scanning calorimetric (DSC) analysis

A MDSC 2920 differential scanning calorimeter (TA Instruments, New Castle, DE, USA) equipped with a refrigerated cooling accessory was used. Calibration was performed using indium as the standard. Samples (15–20 mg) were weighed into aluminum pans, the pans sealed with aluminum lids, and pinholes were made in the lids to allow any volatile materials that evolved from the samples to escape. The samples were heated past *Tg*, under nitrogen purge, at a rate of 10 °C/min over the temperature range of 25–120 °C, then cooled at ~10 °C/min and reheated again at the same rate and the temperature range to measure the *Tg* in the second scan. The *Tg* reported is the midpoint of the baseline deflection in the DSC curve.

2.7. Protein characterization

2.7.1. Osmolarity and turbidity measurements

Osmolarity was determined using freezing-point technique by a Semi-Micro Osmometer, model K-7400 (Knauer, Berlin, Germany). A 290 mOsm/kg standard was used as a reference and water as a control. The presence of insoluble aggregates in formulations of rhEGF was assessed by measuring the absorbance at 350 nm. The solution was transferred to a 1-cm pathlength cuvette and immediately measured by using a U-3300 spectrophotometer (Hitachi, Tokyo, Japan), using water for injection as a reference.

2.7.2. Reversed-phase high performance liquid chromatography (RP-HPLC)

A HPLC system, equipped with two L-7100 pumps, a D-7455 diode-array detector, a L7350 column oven and a D-7000 interface module was used (Merck Hitachi, Tokyo, Japan). rhEGF and its degradation products were eluted from a Vydac C18 column equipped with a Vydac C18 guard column (Vydac, Hesperia, CA, USA) and detected at 226 nm. For the analyses, two mobile phases were used: A, 0.1% TFA/distilled water; and B, 0.05% TFA/acetonitrile. The injection volume was 500 μL and the flow rate was 1.0 mL/min. Separation was performed using a linear gradient from 20 to 40% B in 30 min at 35 °C. The percentage purity was calculated from the main peaks area divided by the sum of all detected peaks area. The percentage of the lyophilization process recovery by RP-HPLC area (*R*) was calculated using the following equation:

$$R(\%) = \frac{(\text{rhEGF1} - 51 \text{ peak area} + \text{rhEGF1} - 52 \text{ peak area}) \text{ after lyophilization}}{(\text{rhEGF1} - 51 \text{ peak area} + \text{rhEGF1} - 52 \text{ peak area}) \text{ before lyophilization}} \times 100$$

2.7.3. Size-exclusion high performance liquid chromatography (SEC)

Separation was performed with the system described above using a Superdex 75 HR 10/30 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). SEC was conducted in the presence of arginine since rhEGF nonspecifically binds to the HPLC matrix via a hydrophobic interaction [16]. The chromatographic conditions were: mobile phase of 100 mM $NaPO_4$/500 mM Arginine-HCl at pH 6.5, 0.5 mL/min flow rate, 200 μL injection volume, UV detection a 280 nm, 25 °C and run time for 50.0 min. Molecular weights were determined using molecular weight standards: Bovine Thyroglobulin-670 kDa, Bovine Gamma Globulin-158 kDa, Chiken Ovoalbumin-44 kDa, Horse Myoglobulin-17 kDa, Vitamin B-12-1.35 kDa (Bio-Rad Laboratories, CA, USA).

2.7.4. Enzyme-linked immunosorbent assay (ELISA)

The concentration of rhEGF was quantified by a sandwich-type ELISA [17]. The rhEGF contained in the sample was captured by a monoclonal antibody (mAb) coupled to the solid phase, CB-EGF.1, and subsequently detected by adding a second antibody bound to the enzyme (peroxidase-conjugated CB-EGF.2). As reference for quantification, samples were assayed in parallel with serially diluted amounts from 5.0 to 0.313 ng/mL of a working reference material. The percentage of recovery by ELISA (R) after lyophilization was calculated using the following equation:

$$R(\%) = \frac{\text{rhEGF ELISA after lyophilization}}{\text{rhEGF ELISA before lyophilization}} \times 100$$

2.7.5. Biological activity test

The ability of rhEGF to stimulate the proliferation of BALB/c 3T3 A31 murine fibroblast cells (ECACC, Wiltshire, UK) was measured by a colorimetric assay. Cells were seeded at a density of 1.5×10^4 cells/mL in 100 μL/well of DMEM supplemented at 5% fetal bovine serum in 96-well plates. Then, cells were stressed until a quiescence state and incubated with different amounts of rhEGF (diluted in DMEM). Afterwards, the medium was removed and the viable cells were determined by crystal violet staining. Finally, the plate was read at 578 nm on a conventional plate reader. The results were expressed as international units (IU), compared to a secondary reference which was calibrated against the 91/530 EGF standard (NIBSC, Hertfordshire, UK). The biological activity of rhEGF was assessed from the absorbance at 578 nm (A578 nm) by means of a parallel line assay statistical software based on the previously described methodology [18]. The percentage of recovery of bioactivity (R) after lyophilization was calculated using the following equation:

$$R(\%) = \frac{\text{rhEGF bioactivity after lyophilization}}{\text{rhEGF bioactivity before lyophilization}} \times 100$$

2.8. Stability studies

Firstly, the control sample vials, equivalent to zero incubation time, were stored at −70 °C. Following storage, three vials of each sample were pooled at each time point. Just prior to analysis, the lyophilized samples were rehydrated with their original volume of WFI. The tests were performed in triplicate for calculation of the degradation kinetics of rhEGF.

For individual excipients screening, the rhEGF stability was followed by RP-HPLC and ELISA (see above). After storing in a KBF 115 incubator at 50 °C (Binder, Tuttlingen, Germany), the freeze-dried samples were analyzed by RP-HPLC on days 0, 2, 4, 7, 15, 30, 45 and 60; and by ELISA on days 0, 7, 15, 30, 45 and 60. The effect of excipients on the degradation kinetics of rhEGF was determined.

Taking into account the effect of dextran-sucrose mixture on freeze-dried formulations, the rhEGF stability was followed by RP-HPLC, ELISA and bioactivity assays (see above). The freeze-dried samples were analyzed on days 0, 7, 15, 30, 45 and 60. Then, the effect of dextran weight fraction on the degradation kinetic of rhEGF was determined.

The stability of rhEGF in the pre-lyophilized (aqueous) and lyophilized (dried) formulations was studied after storage at 37 °C and 50 °C in KBF 115 incubators (Binder, Tuttlingen, Germany). The protein stability was followed by biological activity and RP-HPLC (see above). The aqueous samples were periodically analyzed by RP-HPLC to determine the area and purity of rhEGF peaks (after 1, 2, 3, 4 and 7 days incubation at 50 °C and after 3, 7, 15, 21 and 30 days incubation at 37 °C). The aqueous samples were also evaluated after 3, 7, 15, 21 and 30 days incubation at 50 °C and after 7, 15, 30, 45, 60, 75 and 90 days incubation at 37 °C by cell proliferation assay. The freeze-dried samples were analyzed by all assays after 7, 15, 30, 45, 60, 75 and 90 days of storage at 37 °C and 50 °C.

3. Results

3.1. Excipients screening

Cryoprotective and/or lyoprotective excipients commonly used in protein lyophilization (sugars, polyalcohols, amino acids and polymers) were tested. As a negative control, a 75 μg/mL rhEGF solution in 10 mM sodium phosphate at pH 7.0 (without any excipients) was freeze-dried and used as a reference.

3.1.1. Excipients effect on formulation attributes

As excipients selection criterion, we combined formulation and process parameters together. Table 1 shows freeze-dry microscopy T_{oc} of the different rhEGF formulations with the lower T_{oc}, below −40 °C obtained for sorbitol and in the absence of excipients (negative control). The highest values (above −15 °C) were obtained for dextran and glycine. Intermediate values were obtained from −27.7 to −35.3 °C for raffinose, mannitol, trehalose and sucrose. In the particular case of mannitol, a wide region of microcolapse was observed from about −29.6 °C to −7.5 °C.

The macroscopic appearance of freeze-dried cakes was not significantly altered during freeze-drying for the majority of the formulations investigated; indicating that drying was conducted below T_{oc} (Table 1). Exceptions were the partially collapsed sucrose and trehalose formulations; and the shrunken cake dimensions found in negative control (without any excipients) and sorbitol formulation. The shrunken cake of sorbitol formulation could be due to the fact that the primary drying was conducted above T_{oc} (−45.9 °C); in the case of negative control it simply did not contain enough solids to create a cake. The macroscopic results were in agreement with the freeze-dry microscopy evaluations and the freeze-drying process. The initial residual moisture levels were similar for both protocols, in the range of 2.8–3.9% and 2.3–2.6% for unnealed and annealed, respectively (Table 1). The larger moisture levels were obtained for negative control, dextran and sugar excipients. Reconstitution times below 1 min were not significantly different among the different formulation cakes. We did not detect differences probably because of the low protein and excipient concentrations, which also tend to render easily reconstituted cakes. Finally, the absorbance at 350 nm was very low for all excipients. In the cases of mannitol and glycine, the different cake quality attributes were similar for both unannealed and annealed freezing protocols.

3.1.2. Excipients effect on rhEGF stability during freeze-drying

To evaluate the stability of rhEGF during freeze-drying in the presence and absence of excipients, RP-HPLC and ELISA were performed, both before and immediately after freeze drying. The RP-HPLC purity of each reconstituted lyophilized formulation was compared to the pre-lyophilized one. The RP-HPLC purity of pre-lyophilized rhEGF concentrated solution was 96.9%. The excipients were considered protective when their standard error of rhEGF purity included 96.9%. As shown in Fig. 1a, purity after freeze-drying was only affected by the negative control. Overall, no relevant differences in purity were observed for all the formulations investigated, with values in the range 96.1–97.3%.

Process recovery by RP-HPLC area and ELISA was considered quantitative when including the 100% value in the range of their standard error. RP-HPLC area recovery demonstrated that over 10% of the original peak area was lost when rhEGF was freeze-dried in the absence of excipients (Fig. 1b). According to the ELISA measurements, 10–20% of the original immunoreactivity was lost when

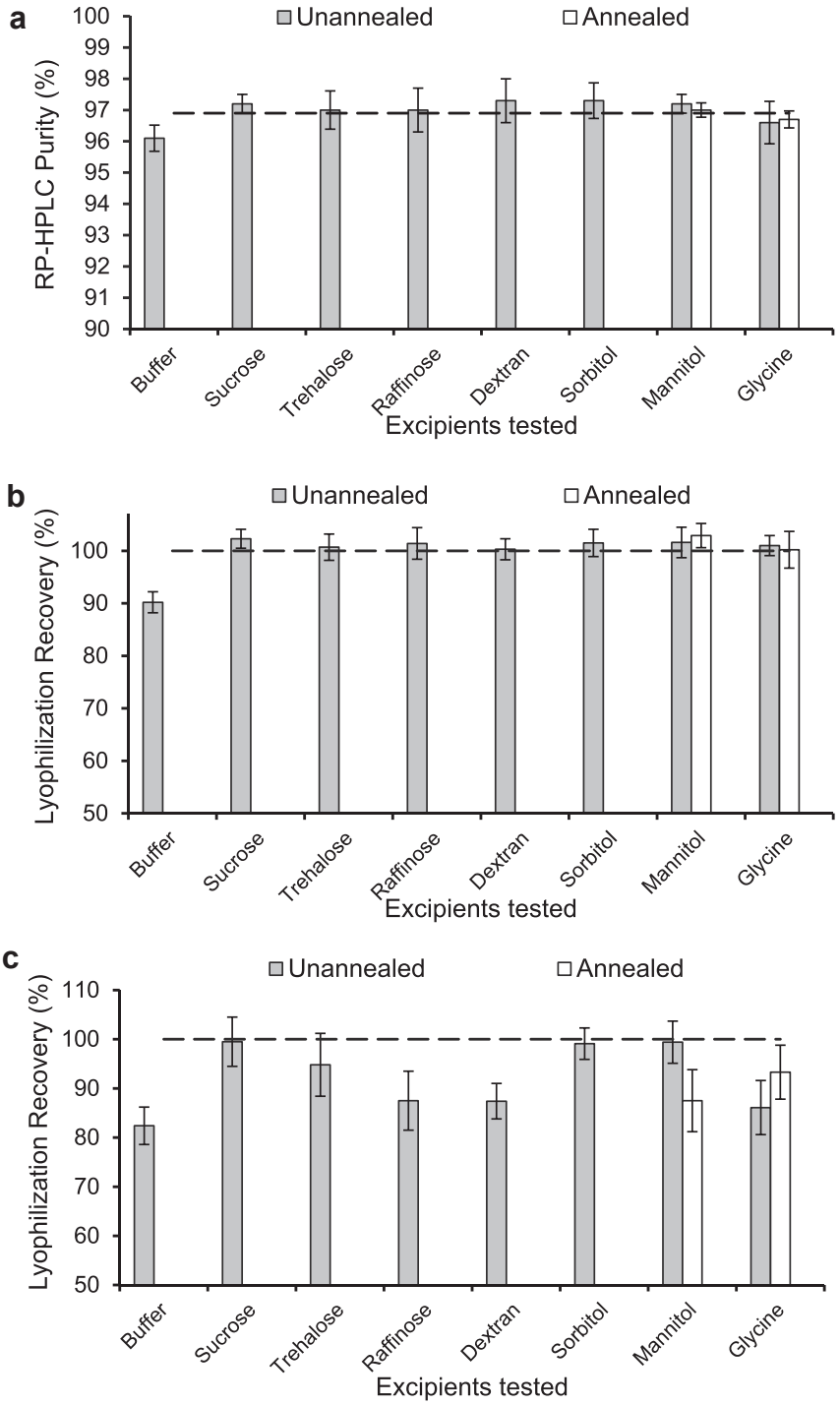

Fig. 1. Influence of individual excipients at 2% (w/v) in 10 mM sodium phosphate buffer pH 7.0 on the stability of rhEGF during the freeze-drying process with unannealed and annealed freezing protocol. Protein stability was assessed by RP-HPLC purity (a) and lyophilization process recovery by RP-HPLC area (b) and ELISA (c) assays.

rhEGF was freeze-dried in the absence of excipients and in the presence of raffinose, dextran and glycine. A similar loss was also obtained in the presence of mannitol with the annealed protocol (Fig. 1c). In contrast, the rhEGF immunoreactivity was not significantly affected when freeze-dried in the presence of sucrose or trehalose (also in mannitol by the unannealed protocol).

3.1.3. Excipients effect on freeze-dried rhEGF stability at high temperature

High temperature is the most widespread method to degrade therapeutic proteins, favoring both aggregation and chemical degradation reactions. Previous reports indicated that the rhEGF has a temperature transition midpoint (T_m) at 55.5 °C [19]. The

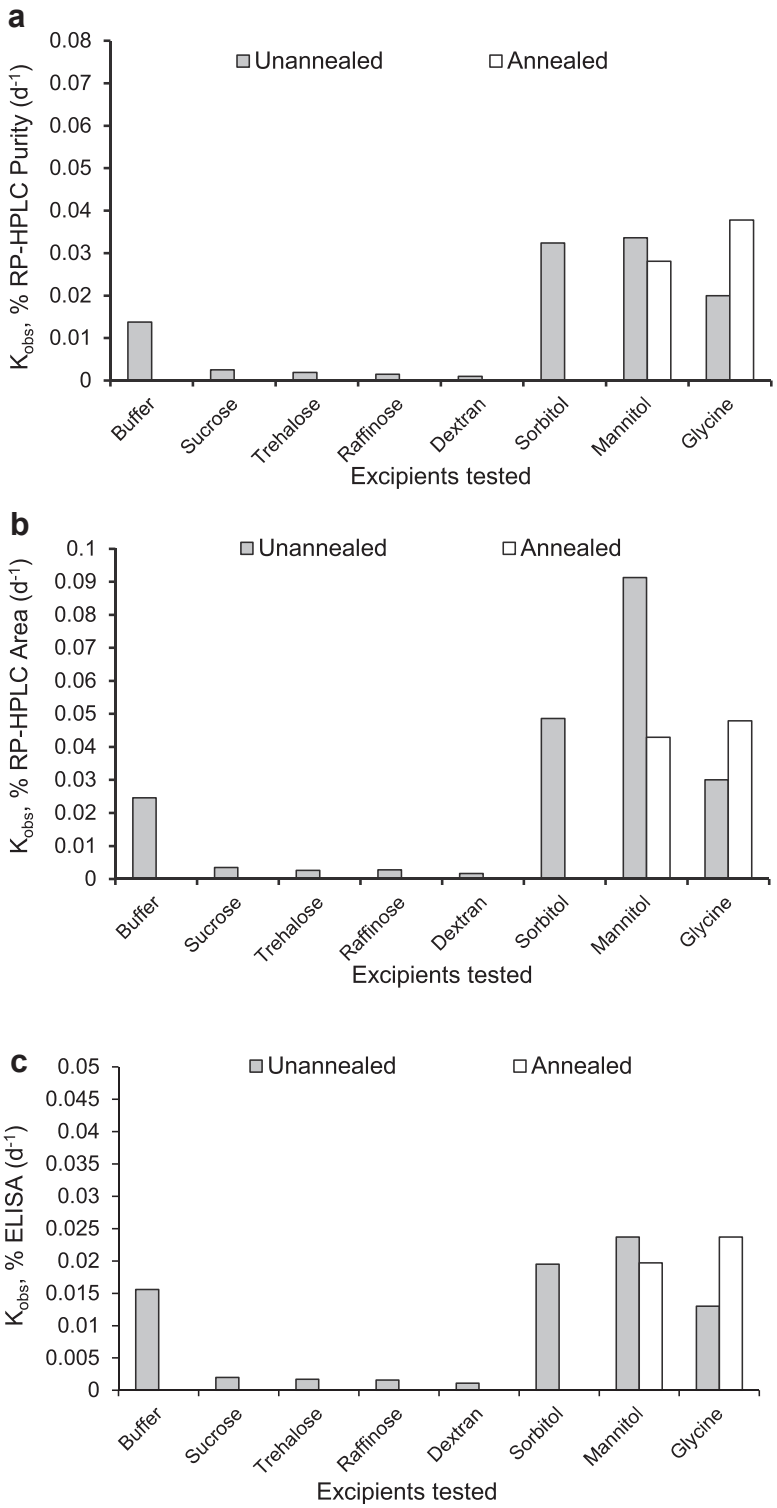

Fig. 2. Excipients effect on degradation rate constants of freeze-dried rhEGF at 50 °C by RP-HPLC purity (a), RP-HPLC area (b) and ELISA (c) assays. Formulations variants, 75 μg/mL rhEGF in 2% (w/v) excipients and 10 mM sodium phosphate buffer pH 7.0.

temperature of 50 °C, near to T_m, was chosen to study degradation kinetics in an extreme condition favoring protein unfolding. The stability of freeze-dried rhEGF in the absence or presence of different excipients was studied after storage at 50 °C. The data obtained from experimental runs demonstrated a linear relationship when ploting ln (percentage remained) *vs.* time, indicating pseudo first-order kinetics by both RP-HPLC and ELISA assays (data not shown). All plots showed correlation coefficients higher than 0.95. rhEGF rate constants (K_{obs}) calculated by the different analytical methods decreased in the following order: RP-HPLC peak area > RP-HPLC peak purity > ELISA (Fig. 2). The mAb-based ELISA assay is less sensitive than RP-HPLC to detect simultaneously the different chemical modifications. This is also the case of RP-HPLC peak area compared to purity because the former might detects changes due to protein adsorption and/or aggregation [13].

The results of the degradation rate constants at 50 °C (Fig. 2), showed the presence of better and worse rhEGF stabilizer excipients. The better stabilizer (sucrose, trehalose, raffinose and dextran) showed K_{obs} below 0.005 days^{-1} while the worse stabilizer (negative control, mannitol, sorbitol and glycine) showed K_{obs} above 0.01 days^{-1}. The degradation rates in formulations with sorbitol, mannitol or glycine were as bad or even worse than in buffer alone.

Fig. 3 shows a typical chromatogram of freeze-dried rhEGF in different excipients after 7 days of storage at 50 °C. The peaks were identified according to previous characterization studies by mass spectrometry [13]. As the starting rhEGF, the freeze-dried and heated in sucrose, dextran, trehalose and raffinose (represented by the sucrose profile) only showed the two rhEGF isomers peak 2 for rhEGF1-51 and peak 5 for rhEGF1-52 with no new or increase in the signal of degradation product peaks detected. The majority of ineffective excipients showed highest signal peaks corresponding to Asn-1 deamidation and Met-21 oxidation for rhEGF1-51 and rhEGF1-52, respectively. In the different freeze-dried formulations the same degradation pathways were detected as previously reported for aqueous conditions [13].

Chromatographic profiles for samples in the presence of glycine show more intense signals corresponding to Met-21 oxidation for rhEGF1-51 and rhEGF1-52, respectively. The same glycine formulations, but in aqueous solution, were assayed by RP-HPLC after 7 days of storage at 50 °C and there were no signals increasing for Met-21 oxidation (data not shown). For sorbitol, two new RP-HPLC peaks corresponding to degradation products were detected: one between peaks 1 and 2, and the other between peaks 4 and 5 (Fig. 3).

Based on the ability of dextran to provide an excellent amorphous bulking agent [20], its high T_{oc} (−14.1 °C) and stabilization performance of freeze-dried rhEGF at 50 °C, a potential formulation containing dextran-disaccharide mixture was proposed for further research because of the high stabilizing effect of disaccharides during freeze-drying process and after storage of dried rhEGF at 50 °C. Since sucrose and trehalose showed similar stabilization performance; sucrose was selected due to its history of use in lyophilized preparations and is less expensive than the alternate trehalose.

3.2. Combined effects of sucrose and dextran

Due to the potential enhancement of the formulation pharmaceutical properties by the dextran-sucrose mixture, their effect on the development of a rhEGF freeze-dried dosage form was evaluated. Several formulations were prepared containing 2% w/v of total solid in 10 mM sodium phosphate buffer at pH 7.0 (Materials and methods). Freeze-dry microscopy studies showed increased T_{oc} with the increase in dextran weight fraction (Table 2). No indication

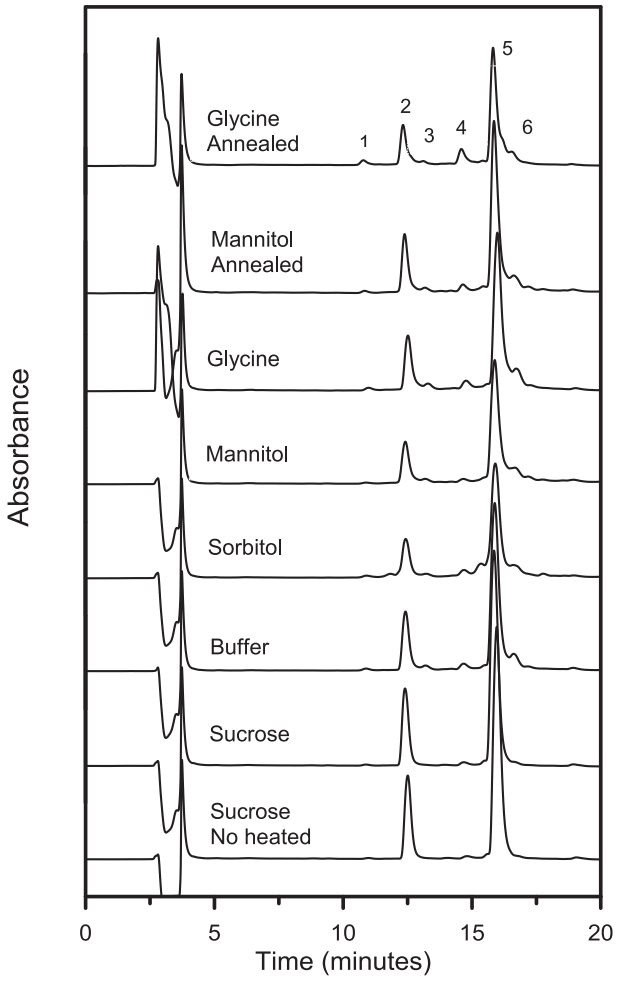

Fig. 3. RP-HPLC chromatographic profiles corresponding to formulation excipients with unannealed and annealed freezing process protocols; samples were assayed after 7 days of exposure at 50 °C. Peak 1: rhEGF1-51 oxidized at Met-21; peak 2: native rhEGF1-51; peak 3: rhEGF1-51 deamidated at Asn-1; peak 4: rhEGF1-52 oxidized at Met-21; peak 5: native rhEGF1-52; peak 6: rhEGF1-52 deamidated at Asn-1.

of skin or crust formations were noticed for different conditions studied. No macroscopic defects were observed except for the formulation using only sucrose, resulting in partially collapsed cake. The reconstitution times were below 20 s and the absorbance at 350 nm was maintained at very low levels, indicating the absence of insoluble particles. The initial residual moisture levels ranged 3.0–4.2% (Table 2).

The RP-HPLC, ELISA and biological activity assays in the reconstituted lyophilizate were compared to the pre-lyophilized one in order to evaluate the effect of dextran-sucrose mixture on rhEGF stability during freeze-drying. No changes in RP-HPLC purity and recovery were detected in any formulation during the freeze-drying (data not shown). Mixtures with weight fraction of dextran up to 0.5 w/w reached a quantitative rhEGF recovery, above 95% in both the ELISA and biological activity assays (Fig. 4a). On the contrary, the recovery was affected in mixtures with weight fraction of dextran above 0.5 w/w. The rhEGF stability after cake exposure to 50 °C increased as well as dextran weight fraction (Fig. 4b). Probably, the inhibition of deamidation and isomerization rates for rhEGF may be due to mobility constraints encountered by the protein in a solid matrix.

Based on previous results, a formulation containing only sucrose was rejected because of its organoleptic properties and lower

stability at 50 °C (Fig. 4b). The formulations with weight fraction of dextran above 0.5 w/w were rejected due to the decrease in rhEGF recovery during the freeze-drying process (Fig. 4a). A formulation containing a weight fraction of dextran of 0.25 w/w, 15 mg/mL sucrose, 5 mg/mL dextran, 10 mM sodium phosphate, pH 7.0 was selected in order to keep a safety margin to the critical dextran weight fractions. Freeze-dry microscopy evidenced a T_{oc} at −30.8 °C for the selected formulation and −27.8 °C was observed as the T_{cc}. For the formulation containing 2% (w/v) using only sucrose, the T_{oc} reached −35.3 °C but the one containing only dextran the T_{oc} obtained was at −14.1 °C (Table 1). Similar T_{oc}, close to −30 °C was obtained at 25 and 250 μg/mL rhEGF concentrations.

3.3. Characterization of freeze-drying and delivery processes effect on rhEGF stability

Due to the complexity of chronic wounds environment [21], the injection solution tonicity should be restricted to a range that mimics the physiological values, 270-330 mOsmol/kg, maintaining the iso-osmolality with the tissue. The selected formulation was designed to achieve iso-osmolality after reconstitution with NSS. Cake reconstitution with NSS gave an osmolality range of 303–319 mOsmol/kg, being an isotonic solution. On the other hand, it is not mandatory to be isotonic because this is a small-volume parenteral (usually 5.0 mL or less) [22]; therefore WFI and NSS were used as reconstitution diluents for drug characterization studies.

Freeze-drying and reconstitution of protein formulations would damage protein chemical, physical, conformational and biological stabilities. Furthermore, the overall effect of the passage of the formulation through the injection system to be used in the clinical study was also evaluated. To address these drawbacks, the rhEGF stability during the freeze-drying, reconstitution and pass through the needle and syringe (simulated injection) processes were evaluated by different analytical methods. To assess the effect of processing on rhEGF stability, samples were lyophilized from selected formulation solution containing rhEGF at 75 μg/mL. After that, cake vials were rehydrated with both solvents and then passed through a needle attached to a syringe. Five main samples were evaluated: prior to freeze-drying solution (pre-lyo), reconstituted freeze-drying cake in both solvents (reconstituted-lyo/WFI and reconstituted-lyo/NSS) and after simulated injection processes (after-NS/WFI and after-NS/NSS).

RP-HPLC chromatograms corresponding to different rhEGF samples evaluated, where two main peaks were separated corresponding to rhEGF1-51 and rhEGF1-52 are shown in Fig. 5a. The RP-HPLC chromatograms profiles corresponding to the different rhEGF

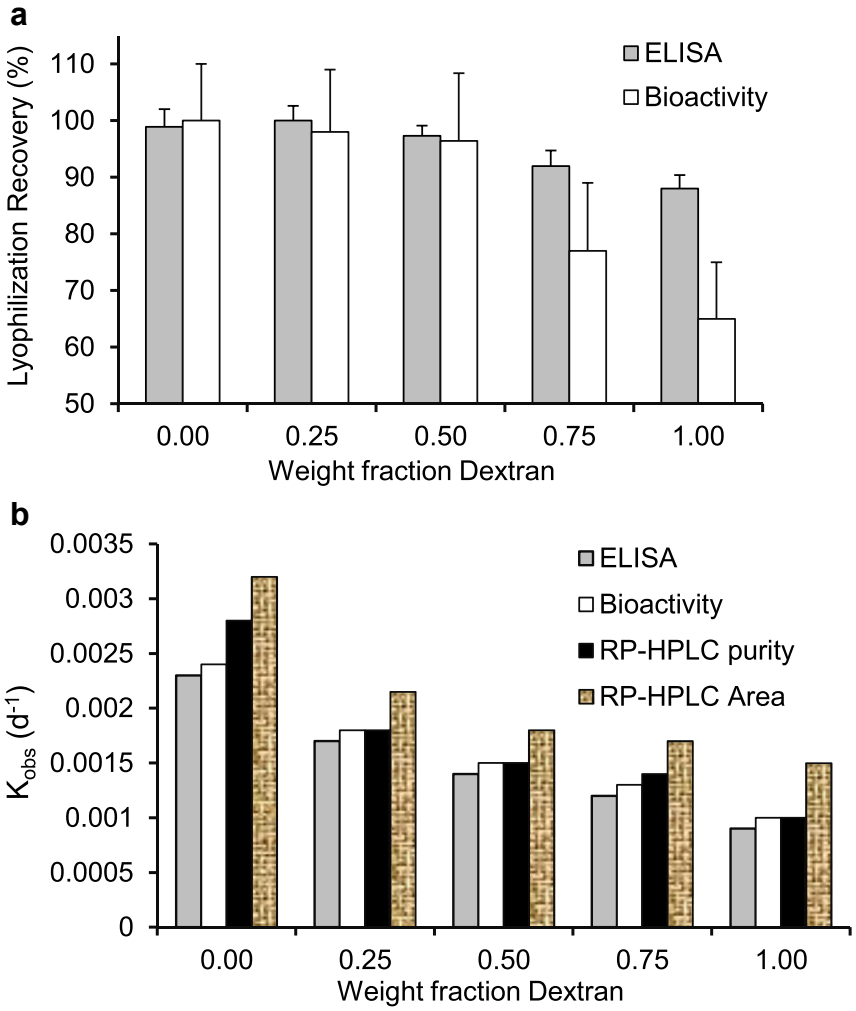

Fig. 4. Influence of dextran weight fraction on the stability of rhEGF in different sucrose-dextran mixtures formulated in 10 mM sodium phosphate buffer pH 7.0. Protein stability was assessed by ELISA, biological activity, RP-HPLC purity and RP-HPLC area assays. (a) Freeze-drying process recovery and (b) degradation rate constant at 50 °C of lyophilized rhEGF.

samples were very similar, showing that neither the freeze-drying nor the simulated injection processes affected the chemical stability of rhEGF. The rhEGF aggregation was evaluated in the different samples by SEC (Fig. 5b). The chromatographic profiles indicated that neither the freeze-drying, nor simulated injection processes by both solvents affected the physical stability of rhEGF. The peak around 29.8 min elution time corresponded to the rhEGF monomer with a molecular weight of 5700 ± 62 g/mol, as determined from the calibration curve (Fig. 5b). The peaks corresponding to the placebo (15 mg/mL sucrose and 5 mg/mL dextran in 10 mM sodium phosphate buffer pH 7.0) could be easily identified by a direct comparison to the rhEGF sample where neither aggregates nor fragments were detected. On the other hand, reducing and non-reducing SDS-PAGE gel samples from pre-lyo, reconstituted-lyo/WFI, reconstituted-lyo/NSS, after-NS/WFI and after-NS/NSS showed an intact full rhEGF band of approximately 6.0 kDa (data not shown).

The rhEGF conformation, as judged by CD spectra, did not differ significantly among samples (Supplementary Fig. S1a). The spectrum above 225 nm was produced primarily by achirality generated by the three intramolecular disulfide bonds, as well as the side-chain chromophores from aromatic residues like tryptophan and tyrosine. The CD profile below 225 nm is indicative of a major contribution from unordered structure [13]. No significant changes were observed in the tryptophan fluorescence emission while comparing rhEGF from samples before freeze-drying, after reconstitution and after the simulated injection processes, indicating the absence of major changes in the local environment of the tryptophan residues (Supplementary Fig. S1b). The normalized spectra of these samples were practically identical and the standard deviation of the maximum emission wavelength values was only 0.85 nm from the average. The emission maximum (λ_{max295}) for the spectrum corresponding to the different samples was from 349 to 351 nm and the half width ($\Delta\lambda_{295}$) from 59 to 60 nm, similar to the values observed by Gallay et al. [23] for rhEGF in sodium acetate at pH 3.6 (λ_{max295} of 349 nm and $\Delta\lambda_{295}$ of 58 nm). The obtained spectrum was typical of highly exposed and flexible tryptophan residues [24], which was consistent with the structural properties displayed by Trp-49 and Trp-50 in the tridimensional structure of human EGF [25]. Therefore, the spectrum showed no indication of protein aggregation.

The biological activity of rhEGF was examined using an *in vitro* cell proliferation assay. The log (concentration) *vs.* response curves followed the familiar symmetrical sigmoidal shape. The goal was to determine the EC50 of the different formulation samples (*i.e.,* the concentration that provokes a response half way between the basal and the maximal response). On the other hand, rhEGF immunoreactivity for the different samples studied was assessed using a conformational mAb-based ELISA as previously described [17,26]. Dose-response curves for cell proliferation and ELISA assay are shown in Supplementary Fig. S2. They were almost identical, indicating that neither the freeze-drying nor the simulated injection processes affected its bioactivity and binding to the two mAb used in the ELISA. Most obviously, rhEGF stability did not decrease after the processing conditions studied, since the same stimulatory effects on the initiation of BALB/c 3T3 cell division were observed. The EC50 of rhEGF in the different samples (pre-Lyo, reconstituted-Lyo and after-NS) in both reconstitutions solvents were in the range 0.50–0.68 ng/mL.

3.4. Temperature stress stability of rhEGF in aqueous vs. freeze-dried formulation

Taking into account that the stability on the dried-state may be influenced by product moisture and *Tg*, the moisture of freeze-

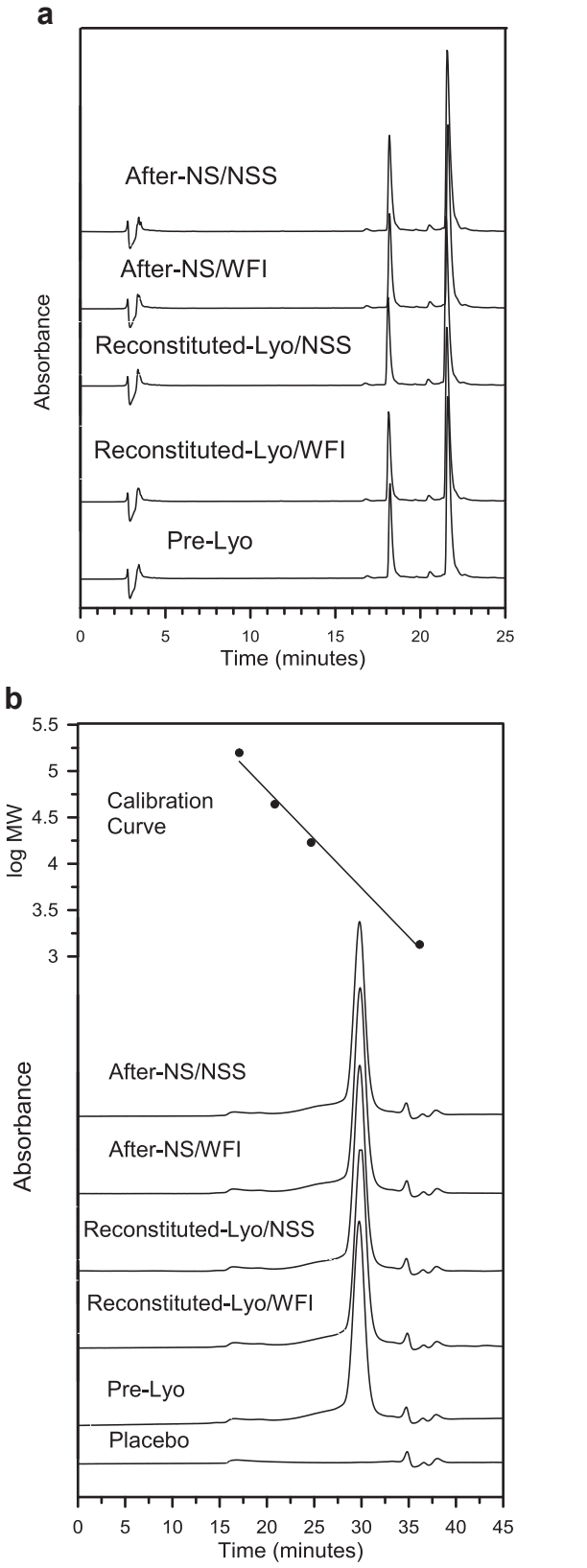

Fig. 5. Effect of freeze-drying and simulated injection processes on rhEGF stability. (a) Overlay of RP-HPLC chromatograms generated at 226 nm and (b) Overlay of SEC chromatograms generated at 280 nm. Freeze-dried samples were reconstituted in WFI and NSS.

dried formulation used in this study was determined by the Karl Fischer's method and its *Tg* by DSC. The dried formulation had 3.1% residual moisture and *Tg* of 52.6 °C. The stability of the formulation was evaluated at about to *Tg* (50 °C) and below the *Tg* (37 °C) and compared with the aqueous formulation at the same temperatures by a cell proliferation assay and RP-HPLC. The samples of lyophilized and liquid formulations were stored at 37 °C and 50 °C for three months.

Degradation kinetics of aqueous and freeze-dried rhEGF formulations by cell proliferation assay and RP-HPLC are showed in Fig. 6. The kinetic was significantly lower in freeze-dried than in aqueous formulation. The degradation rate by cell proliferation assay in aqueous formulation was 0.005 $days^{-1}$ and 0.052 $days^{-1}$ for 37 °C and 50 °C, respectively. For the freeze-dried rhEGF, no significant changes were detected in the degradation kinetic by cell proliferation assay for both temperatures. The degradation rates by RP-HPLC after storage at 37 °C were 0.0333 $days^{-1}$ and 0.0003 $days^{-1}$ for liquid and freeze-dried formulation, respectively. On the other hand, after stress storage at 50 °C, the degradation rates by RP-HPLC were 0.1482 $days^{-1}$ and 0.0021 $days^{-1}$ for liquid and freeze-dried formulation, respectively. The degradation rate in freeze-dried with respect to liquid formulation by RP-HPLC decreased about 100 times at 37 °C and about 70 times at 50 °C. The main degradation product, the Asn-1 deamidation [13], was detected in the RP-HPLC chromatographic profile of aqueous formulation. A high stabilization in the dried formulation was observed in all storage conditions, at temperatures near to or below *Tg*.

4. Discussion

Previous investigations of solution stability and excipient compatibility have highlighted the difficulty in obtaining a stable rhEGF liquid formulation [13]. A solid state formulation is hypothesized as alternative to control rhEGF degradation pathways. Freeze drying formulations are, in general, complex mixtures of excipients that benefit the active ingredient or the product as a whole. However, the exact constitution of a formulation defines the subsequent process parameters. For instance, the lower the critical temperatures of a product, the slower, longer and more expensive freeze drying will be. Therefore, formulation and cycle development were conducted together. That is why, formulation T_{oc}, determined by freeze-dry microscopy, was also considered for excipients selection criterion.

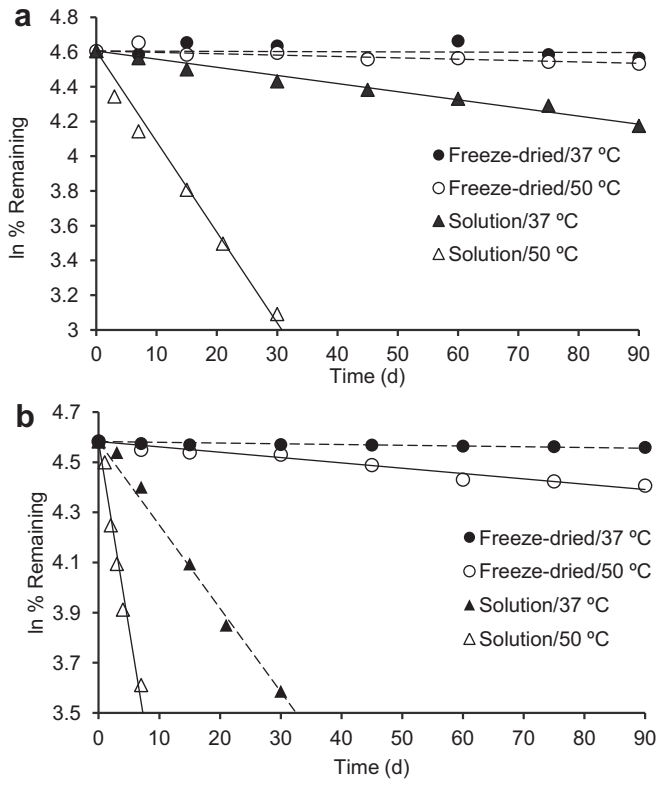

Fig. 6. Stability of selected rhEGF formulation (15 mg/mL sucrose and 5 mg/mL dextran in 10 mM sodium phosphate buffer pH 7.0) in solution and dried state at temperatures of 37 °C and 50 °C by cell proliferation assay (a) and RP-HPLC (b).

In line with other studies, we found that the current measured T_{oc} values were slightly lower than the reported *Tg*′ values for the same excipient formulation (*e.g.* the T_{oc}/*Tg*' in °C for sucrose −35/−32, trehalose −32/−29 and dextran −14/−10) [27,28]. The detection of a wide microcollapse region for mannitol, could be related to amorphous mannitol presence in formulation. Many reports have discussed about the inhibition of mannitol crystallization in the presence of phosphate-containing buffers formulations [29]. Because the residual moisture levels (3–4% w/w) were near to the limit of 3% w/w for therapeutic products [30], robust freeze-dried formulation conditions were explored in the limit of residual moisture, enough to provide an organoleptically acceptable cake with a rhEGF temperature stability significantly improved as compared to the liquid state.

The rhEGF stability was assessed by the previously reported stability-indicative methods: RP-HPLC and ELISA assay. At this point, RP-HPLC was used as the most sensitive assay, instead of the commonest SEC for aggregation studies, to further characterize rhEGF degradation products. RP-HPLC has been used to detect the presence of rhEGF main degradation products (*e.g.*, Met-21 oxidation, Asn-1 deamidation and the conversion of Asp-11 to a stable Asp-succinimide) [13]. The two mAb used in ELISA recognizes only conformational epitopes and, consequently, correctly folded EGF [31]. Specifically, the epitope recognized by the CB-EGF.1 mAb comprises amino acids Ser-9, His-10, Tyr-13, Lys-28, Glu-40 and Arg-41 [26]. This conformational epitope includes the Tyr-13 and Arg-41 residues, essential for the binding of EGF to the EGF receptor and defining the neutralizing activity of the mAb.

Freeze-drying of rhEGF showed that the RP-HPLC and ELISA recoveries decreased once dried in the absence of any excipients. Furthermore, quantitative recovery was achieved by incorporating the protein in disaccharides amorphous matrixes of either sucrose or trehalose. In addition, full stabilization was achieved with mannitol by the unannealed protocol. No general rule was observed between excipient cake collapse and stabilization performance during freeze-drying. Disaccharide formulations were partially collapsed (Table 1), but completely protected rhEGF during freeze-drying. On the contrary, raffinose, dextran and glycine were unable to completely protect protein stability (also mannitol by the annealed one) although they did not collapse. Curiously, reports about the effect of collapse on protein stability after freeze-drying have shown that collapsed cakes have reconstitution times and protein stability comparable to those of non-collapsed lyophilizates [32].

The stability of freeze-dried formulation upon storage at 50 °C was improved by incorporation of rhEGF in amorphous matrix of sucrose, trehalose, raffinose or dextran. The Asn-1 deamidation and Met-21 oxidation degradation pathways were well controlled at this high storage temperature in the presence of these excipients. Reports about the effect of freeze-drying collapse on protein stability during storage at high temperatures have shown full protein stability in collapse cakes [33]. On the other hand, a worse stabilization of lyophilizates that collapse during storage due to the onset of crystallization and hydrolysis of the stabilizer has been reported

[34]. According to our results, no general rules were observed between excipient cake collapse during freeze-drying and stabilization performances at high storage temperature.

Due to the enhanced pharmaceutical properties of dextran-sucrose mixture, these have been used for protein stabilization in freeze-drying [35]. Consequently, the effect of these mixtures on rhEGF freeze-dried dosage form was evaluated. An increase in T_{oc} with the increase in dextran weight fraction was observed for rhEGF mixture formulations (see Table 2). Since dextran has a high Tg' near to −10 °C [27], co-lyophilization of sucrose with dextran increased Tg' and T_{oc} of the formulation allowing higher sublimation temperatures and consequently facilitates, shortens and reduces the process cost [15]. An increase of product temperature by 1 °C decreases primary drying time by approximately 13% [36]. In this sense, the increase on T_{oc} for the selected formulation, about 5 °C with respect to the sucrose, would result in a potential 50% reduction in the primary drying time.

As stated, the bioactivity assay and ELISA are protein conformation sensitive [26], quantitative rhEGF freeze-drying recovery was achieved in lower weight fraction of dextran, but reduced at the higher one. A long chain length (*e.g.*, in dextran) can prevent the formation of intermolecular hydrogen bonds between the protein and the sugars, which explains the inability of the larger saccharides to provide protection to the rhEGF protein [37]. These facts could account for the non-protection of rhEGF during lyophilization in formulations with high dextran content. On the contrary, the temperature stability of the freeze-dried rhEGF was greater at higher dextran weight fractions (Fig. 4b). The rhEGF stability in dried state at high temperature could also be influenced by properties of this dried product like the glass transition and crystallization temperatures. The dextran glass *Tg* near to 190 °C [38] was greater than that of the sucrose which was approximately 74 °C [39]. The additions of excipients like dextran raised the *Tg* of the lyophilized solids and the crystallization temperature significantly [40]. These properties also might account for the enhance stabilities of Asx residues against deamidation and isomerization reactions in the solid state [14,34].

The rhEGF dextran-sucrose formulation successfully protected the protein from the potential damage by freeze-drying and simulated injection processes. This was evidenced when assayed by SDS-PAGE, RP-HPLC and SEC where no significant differences in patterns and purity were detected (Fig. 5). The same was shown for conformational and structural stability as judged by CD and Trp intrinsic fluorescence spectroscopy's (Supplementary Fig. S1). The CD profile was similar to that obtained previously for native human EGF [13]. A minimum around 200 nm has been previously associated to the transitions found in random and/or unordered structures [41]. In addition, the slight changes around the minimum at 200 nm are expected for molecules that present an ensemble of random conformations. The fluorescence spectrum was typical of highly exposed and flexible tryptophan residues [24], which is consistent with the structural properties displayed by Trp-49 and Trp-50 in the tridimensional structure of human EGF [25]. All together these observations indicate no major changes in the atomic environment of the Trp residues. Furthermore, no changes in rhEGF mAb-based ELISA immunoreactivity and potency by cell proliferation assay were detected. The EC50 for the different samples were similar to that of reagent grade EGF, EC50 0.6 ng/mL [42].

The dried formulation of rhEGF showed significantly improved stability, evidenced by results from RP-HPLC and cell proliferation assays. In line with other studies, we found that the current freeze-dried rhEGF notably decreased Asn-deamidation in comparison to liquid state as determined by RP-HPLC (about 100 times at 37 °C and 70 times at 50 °C) [43]. This improved stabilization of rhEGF in the amorphous matrix would be due to the addition of dextran to sucrose based formulations which increase the formulation *Tg* and inhibit sucrose crystallization [33,34,38,39]. Furthermore, the results of this work are in line with a recent report on the preparation of redispersible liposomal dry powder for transdermal delivery of rhEGF. It was shown that the free rhEGF stability in buffer solution decreased markedly after 1 month of storage at 25 °C, with 9.7% of the rhEGF remaining. The liposome formulation increased the protein stability in solution; and the rhEGF liposomal dry powders prepared by ultrasonic spray freeze-drying and lyophilization maintained its full stability after 1 month of storage at 25 °C [44].

Our results indicate that the selected solid state rhEGF formulation represents an appropriate technical solution for long-term stabilization. Furthermore, future stability studies should be done, as recommended by the International Conference of Harmonization (ICH) guidelines, to demonstrate that the formulation fulfills regulatory requirements [45].

5. Conclusions

As this research illustrates, to maintain rhEGF chemical and physical stability during freeze-drying and upon storage at elevated temperature, it was important to perform freeze-drying in carbohydrate formulations that tend to form glassy matrix structures. The highest freeze drying recovery was obtained using sucrose or trehalose but the best stability on storage at 50 °C was observed from addition of dextran, sucrose, trehalose and raffinose. A formulation comprising a mixture of sucrose and dextran provided successful stabilization of rhEGF against chemical, physical, conformational and biological degradation during the processes of freeze-drying, reconstitution (WFI and NSS) and passage through needle and syringe. The selected formulation showed an onset temperature for collapse of −30.8 °C. The dried state formulation resulted in much improved stability compared to the liquid state, providing an appropriate technical solution for long-term stabilization. These results show that the technical characteristics of the selected freeze-dried formulation represents a promising approach for stabilizing rhEGF and allow its therapeutic use as a local infiltration drug for wound healing.

Conflict of interest

The authors declare the absence of any conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biologicals.2014.07.005.

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