Pharm Res (2012) 29:3278–3291
DOI 10.1007/s11095-012-0820-7

RESEARCH PAPER

Effects of Excipients on the Chemical and Physical Stability of Glucagon during Freeze-Drying and Storage in Dried Formulations

Wei-Jie Fang • Wei Qi • John Kinzell • Steven Prestrelski • John F. Carpenter

Received: 15 December 2011 / Accepted: 22 June 2012 / Published online: 6 July 2012

ABSTRACT

Purpose To evaluate the effects of several buffers and excipients on the stability of glucagon during freeze-drying and storage as dried powder formulations.

Methods The chemical and physical stability of glucagon in freeze-dried solid formulations was evaluated by a variety of techniques including mass spectrometry (MS), reversed phase HPLC (RP-HPLC), size exclusion HPLC (SE-HPLC), infrared (IR) spectroscopy, differential scanning calorimetry (DSC) and turbidity.

Results Similar to protein drugs, maintaining the solid amorphous phase by incorporating carbohydrates as well as addition of surfactant protected lyophilized glucagon from degradation during long-term storage. However, different from proteins, maintaining/stabilizing the secondary structure of glucagon was not a prerequisite for its stability.

Conclusions The formulation lessons learned from studies of freeze-dried formulations of proteins can be applied successfully to development of stable formulations of glucagon. However, peptides may behave differently than proteins due to their small molecule size and less ordered structure.

KEY WORDS excipients · freeze-drying · peptides · solid states · stability

W.-J. Fang • W. Qi • J. F. Carpenter (✉)
Department of Pharmaceutical Sciences
University of Colorado Denver
Aurora, CO 80045, USA
e-mail: John.Carpenter@ucdenver.edu

J. Kinzell • S. Prestrelski
Xeris Pharmaceuticals, Inc.
San Rafael, CA 94801, USA

Present Address:
W.-J. Fang
Zhejiang Hisun Pharmaceutical Inc.
46 Waisha Rd, Jiaojiang District,
Taizhou, Zhejiang 318000, China

ABBREVIATIONS

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature (*Eur. J. Biochem.* 1984 138, 9–37)

CD	cyclodextrin
DSC	differential scanning calorimetry
ESI-MS	electrospray ionization-mass spectrometry
HES	hydroxylethyl starch
IR	Infrared
PES	polyethylstyrene
PS	polysorbate
RP-HPLC	reversed-phase high-performance liquid chromatography
SE	size exclusion
T_g	glass transition temperature
t_R	retention time

INTRODUCTION

Peptides have become increasingly important as therapeutic products. Currently there are more than 60 approved peptide drugs, and an additional 130 peptide candidates are in clinical development (1). As is the case with protein therapeutics, peptide drugs are susceptible to both chemical and physical degradation, and stabilization of these products is challenging (2–5). One approach to achieving sufficient stability of a biological product is to develop a freeze-dried formulation (6,7). There is an extensive literature describing the capacities of various excipients to stabilize proteins during the freeze-drying process and long-term storage in dried formulations, as well as the mechanisms for such stabilization (6–8). For peptide drugs there is a more limited literature on stabilization in freeze-dried formulations (9–12). Therefore, the goal of the current study was to gain further

insights into how the lessons learned from protein therapeutics apply to development of stable freeze-dried formulations of peptide drugs.

Typically, an essential stabilizer in a freeze-dried protein formulation is a nonreducing disaccharide such as sucrose or trehalose (6,7). These sugars can inhibit protein unfolding during the freezing and drying steps of freeze-drying, as well as provide a glassy matrix that is important for long-term storage stability of the dried product (6,7). For drugs that are formulated at acid pH, sucrose has the disadvantage of being susceptible to acid-catalyzed hydrolysis forming reducing sugars glucose and fructose,(13) which can chemically degrade proteins or peptides via the Malliard reaction (13). For example, the Malliard reaction has been reported when glucagon was formulated with the reducing disaccharide lactose (14). Trehalose is more resistant to acidic hydrolysis than sucrose and is more suitable for formulations at low pH (15,16).

The inclusion of a polymer such as hydroxylethyl starch (HES) to a lyophilized protein formulation may improve long-term storage stability of the protein in formulations that also contain sucrose or trehalose (17). The increased stability is observed because HES can form glassy matrix with very high T_g (i.e. >200°C). However, HES alone usually fails to confer stability to dried proteins because it does not inhibit protein unfolding during freeze-drying, and the rate of degradation during storage is greatly increased for unfolded proteins (6,7,17).

The non-ionic surfactants polysorbate 20 and 80 have been used extensively as excipients in freeze-dried and aqueous solution formulations of proteins due to their ability to reduce protein aggregation (18–20). This protective effect has been attributed to several different mechanisms including competing with protein molecules for interfaces, increasing thermodynamic stability of the native state through binding to the protein, fostering refolding and reducing the concentration of protein molecules in a stagnant boundary during rehydration of dried formulations (20). Cyclodextrins also have been shown to reduce protein and peptide aggregation by competing for the air-water interface (21) and by binding to the hydrophobic residues of proteins or peptides such as glucagon (14,22).

The potential stabilizing effects of each of these classes of protein stabilizers for small peptides in freeze-dried formulations are not well understood. Among therapeutic peptides, insulin has been most studied in dried formulations. Although insulin is different from most other peptides in that it has relatively well-defined tertiary and quaternary structures, it is still instructive to consider results for it in the broader context of peptide stabilization. Previous work has shown that insulin in freeze-dried formulations exhibited many of the same pathways of degradation (i.e. aggregation and deamidation) as in aqueous solution (9,10). In one study, the degradation rate was dependent on the pH of aqueous solution prior to freeze-drying as well as the water content of the freeze-dried cake (9,10). Covalent dimerization of human insulin was substantially decreased by incorporation into a glassy matrix of trehalose, presumably by inhibiting structural perturbation of the peptide, reducing molecular mobility in the dried formulation and physically separating the insulin molecules (10). In another study, insulin freeze-dried with trehalose exhibited a substantially lower local dynamics (β-relaxation) and lower degradation rates (i.e. deamidation and dimerization) than insulin freeze-dried with dextran (12). The authors speculated β-relaxation of insulin was reduced because of hydrogen bonding with trehalose, and thus chemical degradation was inhibited. On the other hand, dextran cannot form hydrogen bonds with insulin as readily due to steric hindrance, and therefore reduction of β-relaxation was not present in the dextran formulation.

The study of stability in freeze-dried formulation with other peptides is rather limited. For example, in one study, the level of mannitol freeze-dried formulations of atrial natriuretic peptide (ANP) affected the amount of multimers formed during storage. The authors speculated that in the less stable formulations, which had higher mannitol levels, mannitol crystalized and increased the water moisture content in the amorphous phase (11).

Glucagon (Fig. 1), the focus of the current study, is a polypeptide hormone composed of 29 amino acid residues that is currently used for the emergency treatment of insulin-induced hypoglycemia (23). Glucagon is known for its propensity to degrade both chemically (i.e., hydrolysis and oxidation)(24–26) and physically (i.e., aggregation)(27,28); including formation of aggregates during freeze-drying and rehydration in the absence of stabilizing excipients (29). Therefore, it is an excellent model peptide to evaluate the effects of different buffers, the surfactant polysorbate 20, trehalose, HES and β-CD on stability during freeze-drying and storage in dried formulations.

MATERIALS AND METHODS

Materials

All chemicals were of reagent grade or higher quality. Glucagon was purchased from the American Peptide Company (Sunnyvale, CA). β-CD was purchased from Cyclodextrin Technologies Development, Inc. (High Springs, FL). Trehalose dihydrate was purchased from J.T.Baker (Philipsburg, NJ). HES (Viastarch, MW 200KDa) was purchased from Fresenius (Graz, Austria). Phosphoric acid, citric acid, glycine, 2M hydrogen chloride, sodium chloride, sodium hydroxide, acetonitrile, hydrogen peroxide, potassium

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-**Asp**[9]-Tyr-Ser-Lys-Tyr-Leu-**Asp**[15]-Ser-Arg-Arg-Ala-Gln-

Asp[21]-Phe-Val-Gln-Trp-Leu-**Met**[27]-Asn-Thr

Fig. 1 Primary structure of glucagon. The three Asp and Met residues are shown in bold.

bromide and polysorbate 20 were purchased from Fisher Scientific (Hampton, NH). Nylon membrane filters (0.45 μm) were purchased from Whatman (Maidstone, England). Polyethylstyrene (PES) membrane filters (0.2 μm) were purchased from Millipore (Billerica, MA). 3-ml lyophilization vials (borosilicate glasses, type 8412-B) and gray butyl stoppers (Product # 10123524) were purchased from West Pharmaceutical (Lionville, PA). Other reagents and chemicals were purchased from Sigma-Aldrich (Milwaukee, WI).

Acidic Degradation of Glucagon in Aqueous Solution

Glucagon (0.5 mg/ml) was dissolved in 5 mM sodium phosphate buffer (pH=3.0) and incubated for 5 days at 60°C. Aliquots (0.5 mL) of the solution were removed at various times and centrifuged at 14,500 rpm for 10 min. The supernatant was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) as described below. Samples were also analyzed by electrospray ionization-mass spectrometry (ESI-MS; see below).

Hydrogen Peroxide-Induced Oxidation of Glucagon in Aqueous Solution

We followed the USP method (USP 30 Official Monograph for Glucagon, 2007) for forced oxidation of glucagon. Glucagon (1 mg/ml) was oxidized by incubation in 0.6% hydrogen peroxide (H_2O_2), 20% acetonitrile and 80% water with 10 mM HCl at 4°C for 210 min. Aliquots were removed at various times and analyzed by RP-HPLC using the method described below. The oxidation products were also analyzed by ESI-MS as well as tandem MS (MS-MS; see below).

ESI-MS and MS-MS Analysis of Glucagon and its Degradation Products

Glucagon and its degradation products were analyzed with an electrospray-triple quadrupole-time-of-flight mass spectrometer (ESI-qTOF-MS) from Applied Biosystems (PE SCIEX/ABI API QSTAR Pulsar i Hybrid LC/MS/MSESL). Mass Spectra were acquired by scanning a mass-to-charge ratios (m/z) range from 100 to 2000. Eluates (1 mg/ml glucagon, 50 μl) were injected into the mass spectrometer at a flow rate of 5 μl/min. Spray voltage was set at 4500 V, and capillary temperature was set at ambient temperature. For MS-MS experiments on glucagon and oxidized glucagon, various m/z components were selected and fragmented with suitable collision energy to have reasonable amount of peaks following fragmentation.

Sample Preparation for Freeze-Drying

Glucagon (10 mg/mL) was dissolved in three different buffers (glycine hydrochloride, sodium phosphate, and sodium citrate buffers, 5 mM, pH 3.0). The solution was then mixed in a 1:1 (v/v) ratio with various excipient solutions (prepared at twice the desired concentration using corresponding buffer) to obtain a final glucagon concentration of 5 mg/mL and the final desired excipient concentration of 0.01% for polysorbate 20 and 10 mg/mL for the carbohydrates. The solution was then filtered through 0.2 μm Millipore PES membrane. Sample preparation was conducted in a 4°C cold room. The glucagon concentration and the purity were determined by RP-HPLC (see below).

Freeze-Drying

The formulations were pipetted (0.3 mL) into 3-ml lyophilization vials (13-mm ID) and freeze-dried in a FTS Durastop freeze-drier (Stoneridge, NY). For freezing, samples were cooled to −40°C at 2.5°C/min and maintained at this temperature for 2 h. Then the shelf temperature was increased to -5°C at 2°C/min and held for 2 h as an annealing step (29). The temperature was then decreased to −30°C at 1.5°C/min and the chamber pressure was reduced to 8 Pascal. These conditions were maintained during 24 h for primary drying. Then the shelf temperature was increased to 40°C at 0.5°C/min and held at 40°C for 10 h for secondary drying. Then, the vials were stoppered under vacuum using gray butyl stoppers. None of the formulations showed any visual evidence of cake collapse following freeze-drying.

Storage Studies

Following freeze-drying, the sample vials were incubated at 60°C and analyzed after 2 weeks. Triplicate sample vials for each formulation were incubated. Control sample vials (equivalent to time zero of incubation) were stored at −80°C until analysis. Following storage, formulations were first rehydrated to 5 mg/mL with water and then diluted to 1 mg/mL glucagon with the corresponding buffer. An aliquot of the rehydrated sample (200 μL) was analyzed for

turbidity (see below). Then, the solution was centrifuged (14,500 rpm×10 min) to removed insoluble material. The supernatant was diluted 1:1 with the corresponding buffer for RP-HPLC and size exclusion (SE)-HPLC analysis (see below).

Differential Scanning Calorimetry (DSC)(30)

The glass transition temperature (T_g) of dried formulations was determined using a Perkin-Elmer Pyris-1 DSC (Norwalk, CT). Prior to sample analysis, the instrument was calibrated for melting temperature and heat of fusion with indium (onset of melting: 156.6°C; heat of fusion: 28.45 J/g). In a dry box, the sample vials were opened and about 1 mg samples of dry powder were placed in hermetically sealed aluminum pans. DSC thermograms were collected from 25°C up to 250°C (depending on the compositions of formulations) at a heating rate of 100°C/min. Baselines were determined using an empty pan, and all thermograms were baseline corrected. For measurement of T_g, the samples were first heated to above T_g to remove thermal history, and then cooled back to 25°C and rescanned at 100°C/min. The thermogram obtained during the second scan was used to measure T_g which was determined as the midpoint of the transition.

Infrared (IR) Spectroscopy

Infrared spectra of glucagon in aqueous solution and in freeze-dried formulations were collected at room temperature using a Bomem Prota spectrometer (Quebec, Canada) purged with dry nitrogen (31,32). Spectra of glucagon in aqueous solution were obtained at a peptide concentration of 20 mg/ml (5 mM sodium phosphate buffer, pH 3.0) in a 6 μm pathlength cell with CaF_2 windows. For each sample, a 32-scan interferogram was collected in the single-beam mode with a resolution of 4 cm^{-1}. Peptide spectra were processed to substract absorbance from water vapor and the buffer spectrum as previously described (31,32). For IR spectra of freeze-dried samples, a mass of dried formulation containing about 1 mg of glucagon was mixed with 500 mg potassium bromide powder. The mixture was ground gently and then annealed into a pellet using a hydraulic press (30). The spectra were transformed to second derivatives using Bomem Grams/32 software. The final protein spectra were smoothed with a seven-point Savitsky-Golay function. For comparison of spectra they were area normalized in the amide I region (1600–1700 cm^{-1}) and overlaid (30).

Optical Density at 405 nm (OD_{405})

The presence of aggregates in rehydrated formulations (1 mg/mL glucagon) was assessed by measuring the OD_{405} using a Molecular Devices microplate reader (Sunnyvale, CA). The freeze-dried glucagon formulations were first rehydrated with water to 5 mg/mL glucagon and then diluted with corresponding buffer to 1 mg/mL peptide concentration. Prior to analysis, the plates were shaken for 10 s to mix the solutions in the wells.

Reversed Phase-High Performance Liquid Chromatography (RP-HPLC)

Chemical degradation of glucagon formulations was quantified by RP-HPLC, using an Agilent 1100 HPLC system (Agilent Technologies, Inc., Waldbronn, Karlsruhe, Germany) with a Thermo Biobasic C8 column (5 μm, 250× 4.0 mm ID, Waltham, MA) and a Restek VWD G1314A detector (Bellefonte, PA). The mobile phase was 73% phosphate-cysteine buffer (pH 2.6) with 27% acetonitrile (USP 30 Official Monograph for Glucagon, pp 2230–2231, 2007). Before use, mobile phase was filtered with 0.45 μm Nylon membrane filters and degassed. The flow rate for the analysis was 1 ml/min, and elution was monitored at 214 nm. The temperature of the column was maintained at 37°C. The peak areas of glucagon and its chemical degradation products were used to determine the chemical degradation of glucagon occurring during freeze-drying and storage.

Size Exclusion-HPLC (SE-HPLC)

Aggregation of glucagon in different formulations was determined with SE-HPLC, using an Agilent 1100 HPLC equipped with a TSK $G2000SW_{xl}$ gel filtration column (5 μm, 300×7.8 mm ID) and a Restek DAD G1315A detector. The mobile phase was 3.2 mM HCl, 100 mM sodium chloride, pH 2.5. Before use, the mobile phase was filtered and degassed. The flow rate for the analysis was 1 ml/min and elution was monitored at 280 nm. The percent of monomer remaining in formulations was calculated based on the monomer area of the formulation samples compared to that for liquid control sample. Soluble aggregates were at minimal levels (<0.5%) in all of the samples (data not shown).

RESULTS AND DISCUSSION

Accelerated Acid Degradation of Glucagon in Aqueous Solution

Glucagon has an isoelectric point of about 7 and has a high solubility at pH values less than 3 or greater than 9. The recommended pH range for solutions of the peptide is between 2.5 and 3.0 (US pharmacopeia 24, 1999).

However, as in the case with many peptide and protein drugs, glucagon is susceptible to acid-catalyzed degradations (i.e. Asp cleavage) (24–26). To develop an assay to characterize acid-catalyzed hydrolysis of glucagon, the peptide was first incubated in 5 mM sodium phosphate buffer (pH=3.0) at 60°C and characterized as a function of incubation time. The commercial glucagon we used had some impurities prior to incubation (about 8–9%, see Fig. 3a and its enlarged version Fig. 3b). During incubation, glucagon underwent significant further chemical degradation, with 50% loss of the parent peptide after incubation for 5 days (Figs. 2 and 3d). As expected in the acidic conditions, the main degradation products were due to Asp cleavage, as confirmed by ESI-MS analysis (Table I). Glucagon has three Asp residues and, therefore, six main fragments were observed in ESI-MS.

In RP-HPLC (Fig. 3d), four main peaks (retention time (t_R) around 7, 14, 19 and 29 min, respectively) and several smaller peaks (i.e. t_R around 33, 37, 40, and 42 min) were observed. The main peaks were probably due to Asp cleavage whereas the smaller peaks could be due to Asp cleavage and/or deamidation (25,26). Glucagon has three Gln and one Asn residue. Deamidated residues were not specifically identified in the current study.

Accelerated Oxidation of Glucagon in Aqueous Solution

Although oxidation of Met does not impact the binding affinity of glucagon to its receptor (33), the potency the oxidized peptide in stimulating glucose production in isolated rat adipocytes and hepatocytes is decreased (34). Therefore, oxidation of the peptide should be monitored and minimized. To develop an assay for oxidized glucagon, the peptide was incubated in 0.6% (v/v) hydrogen peroxide at room temperature (Fig. 4). Samples were removed as a function of time and analyzed by RP-HPLC. Two new peaks with similar t_R and almost identical areas were observed (t_R around 8–9 min, Fig. 3c). Almost all of the parent peptide had decomposed after 210 min of incubation (Fig. 4). The oxidation products had molecular weights of [Glucagon+16] (calculated mass=3498.6; observed: $[M+2H]^{2+}$=1750.2, $[M+3H]^{3+}$=1167.1, $[M+4H]^{4+}$=875.6, $[M+5H]^{5+}$=700.7), suggesting one residue of glucagon was oxidized. MS/MS analysis (Table II) of the oxidation products showed the Met residue at position 27 was oxidized. The two peaks in the RP-HPLC chromatograms were probably due to two diastereomers formed with oxidation of the Met residue. Even though glucagon also has several other amino acids susceptible to oxidation (e.g. His, Trp, Tyr, and Phe), no degradation of these residues by hydrogen peroxide was observed.

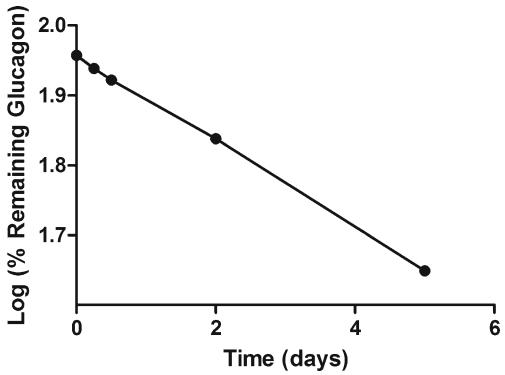

Fig. 2 Degradation of glucagon in 5 mM sodium phosphate buffer pH 3.0 at 60°C. The % remaining glucagon was determined from chromatograms obtained by RP-HPLC. Error bars indicating SD are smaller than the symbols.

Effects of Buffers and Excipients on Glucagon's Stability in Freeze-Dried Formulations

To determine formulation effects on stability of freeze-dried glucagon formulations, we evaluated effects of three buffers (glycine hydrochloride, sodium phosphate and sodium citrate buffers, 5 mM, pH 3.0), polysorbate 20 and three carbohydrate excipients, trehalose, HES and β-CD. An accelerated degradation condition (storage at 60°C for 2 weeks, followed by rehydration) was used to evaluate the stability of the peptide in the freeze-dried formulations. Important physical properties (peptide secondary structure and T_g) were also characterized for each freeze-dried formulation.

The T_g of the freeze-dried formulations was determined by DSC (Table III). When there was no excipient, the T_g of dry powders was between 122°C and 140°C, depending on the buffer used in formulation (Table III). As expected, the presence of polysorbate 20 (0.01%w/v) did not have much effect on T_g.

For formulations with glass forming carbohydrates, the T_g was highest for HES and β-CD, followed by the mixture of trehalose and HES, and then trehalose alone (Table III). These values were consistent with the T_g of the corresponding pure carbohydrates and their mass ratios in the formulations. The buffer also had a substantial effect on T_g, especially in the cases where HES and β-CD were used as excipients (Table III). The cause(s) of the buffer effect was not investigated further.

Infrared spectroscopic analysis of glucagon formulations was used to evaluate the effects of freeze-drying and storage on glucagon's secondary structure, by comparing spectra for the peptide in the dried formulations to that for the native peptide in aqueous solution. The main secondary structure of native glucagon is a mixture of α-helix and random coil (35,36). Correspondingly, the infrared spectrum for native glucagon in aqueous solution has main absorbance around

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