Drug Development and Industrial Pharmacy, 31:135–149, 2005

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1081/DDC-200047368

Taylor & Francis
Taylor & Francis Group

Supercritical Fluids Processing of Recombinant Human Growth Hormone

Sitaram P. Velaga and Johan Carlfors
Department of Pharmacy, Uppsala University, Uppsala, Sweden

ABSTRACT The aim of the study was to investigate the feasibility of precipitating recombinant human growth hormone (hGH) from aqueous solutions using conventional and modified techniques of solution-enhanced dispersion (SEDS) by supercritical fluids. The study investigated the effect on hGH stability of adding isopropanol either as a cosolvent with the original aqueous protein solution (conventional process) or to the supercritical carbon dioxide before mixing with the aqueous protein solution (modified process). The influence of the addition of sucrose (with or without isopropanol) on the precipitation behavior and stability of the protein was also studied. Experiments were performed under various processing conditions (pressure 100–200 bars and temperature 40–50°C), and with various flow rates and solution compositions (CO_2/isopropanol and protein solution). Bioanalytical characterization of the resulting powders involved spectrophotometry, sodium dodecyl sulfate-polycrylamide gel electrophoresis, reverse-phase high performance liquid chromatography (RP-HPLC), and size exclusion chromatography. Solid-state characterization was performed using differential scanning calorimetry, X-ray powder diffraction, scanning electron microscopy, and Karl Fischer techniques. Results showed that with both conventional and modified methods, under optimum processing conditions, the presence of sucrose in the solution decreased the destabilizing effects of the solvent and/or process on the structure of hGH. More hGH was dissolved from the precipitated powders containing sucrose than from those containing only isopropanol. Reverse-phase HPLC indicated that about 94% of the hGH was recovered in its native form. The proportion of dimers and oligomers was reduced in the presence of sucrose; about 92% of the soluble protein was present in monomer form under optimal conditions. The remaining undissolved protein was in monomeric form. The precipitated powders were amorphous, containing particulate aggregates in the size range 1–6 μm with 5–10% residual moisture content. In conclusion, hGH was successfully precipitated from aqueous solution using SEDS technology. The presence of sucrose in the protein solution promoted the precipitation of hGH and reduced aggregation and improved dissolution.

KEYWORDS Supercritical fluids, SEDS, Human growth hormone, Precipitation, Powders, Protein, Stability

Address correspondence to Johan Carlfors, Department of Pharmacy, Uppsala University, Box 580, Uppsala SE 751 23, Sweden; Fax: +46-18-4714223; E-mail: johan.carlfors@farmaci.uu.se

INTRODUCTION

In the development of therapeutic proteins, it is vital to design a formulation that is stable during manufacturing, shipping, and long-term storage. Proteins are preferably formulated in aqueous solution. However, many proteins are susceptible to chemical (e.g., deamidation or oxidation) and/or physical degradation (e.g., aggregation and precipitation) in aqueous solution (Arakawa et al., 1993; Manning et al., 1989). In the dried state, degradation reactions can be avoided or sufficiently retarded to retain the stability of the protein formulation for longer periods at ambient temperatures (Pikal, 1994). Current methods for preparing solid protein formulations include freeze-and spray-drying. Lyophilization (freeze-drying) is more common, but it is a long, sophisticated, and expensive process. In addition, lyophilization can generate a variety of stresses due to freezing, drying, pH changes, and phase separation, which subsequently change the structure of the protein (Allison et al., 1998). Spray-drying processes have shown some promise in generating inhalation powders containing antibodies, enzymes, and insulin in recent years. However, the higher temperatures required for thermal dehydration is a major limitation with this methodology (Maa et al., 1998).

Consequently, there is a need for exploring alternative processes that are preferably single-step, continuous, and environmentally benign. Particle precipitation processes using supercritical fluids (SF) have been studied for a variety of compounds to date (Krukonis, 1984; Loth & Hemgesberg, 1986). Since the techniques using SF involve a single step and are relatively simple operations (Debenedetti et al., 1993; Randolph et al., 1993), these methods may emerge as potential alternatives to traditional methods of protein processing. Supercritical fluid processing also results in a sterile product, which provides an additional advantage (Dillow et al., 1999). In particular, gas antisolvent (GAS)-based SF processes have shown significant promise in the preparation of microparticulate protein material from organic solvents (Yeo et al., 1993).

Solution-enhanced dispersion (SEDS) by supercritical fluids a GAS process, involves the co-introduction of a protein solution and an SF through a coaxial nozzle into a particle formation vessel. Dispersion and rapid mixing of the SF with the drug or protein solution are followed by rapid supersaturation and subsequent nucleation and formation of drug/protein particles from a single equilibrium phase (York, 1995). Carbon dioxide (CO_2) is commonly used as a SF in various methodologies, including SEDS, because of its relatively easily attained critical parameters. The SEDS technique, which is used to process small molecules, has been successfully employed in the processing of lysozyme (Moshashaee et al., 2000). However, in SF methods, it is often difficult to find a solvent that dissolves the protein without influencing its stability (Jackson & Mantsch, 1991; Yeo et al., 1993).

While aqueous solutions of the protein are preferable in SF processes, the poor miscibility of water and supercritical carbon dioxide (SC-CO_2) often limits the process. However, the extraction of water during precipitation is possible if polar SF-miscible solvents such as ethanol, acetone, or isopropanol are added to the aqueous solution of the protein (traditional process) or to the SC-CO_2 before it is mixed with the protein solution (modified process) (Bustami et al., 2000). While both these versions of the SEDS process incorporate such a solvent, in this modified version the protein is maintained in an aqueous environment, before the rapid extraction of water into the SC-CO_2. This minimizes the contact of the macromolecule with the organic solvent, thereby retaining its biological activity (Moshashaee et al., 2003; Palakodaty et al., 1998).

Supercritical fluid processing for protein precipitation has mainly been focused on model proteins such as trypsin, albumin and lysozyme (Moshashaee et al., 2003; Sellers et al., 2001; Winters et al., 1996). Recently, however, Nesta, Elliot, and Warr studied the precipitation of recombinant human immunoglobulin from aqueous solutions using a SEDS process (Nesta et al., 2000). Recombinant human growth hormone (hGH), a therapeutic protein, has not been investigated using these technologies, and was chosen for this study.

Recombinant hGH has 191 amino acid residues and a molecular weight of 22 kD; it is identical to the growth hormone of pituitary origin. Currently, hGH is administered subcutaneously for treating growth hormone deficiency disorders. Chemical decomposition of hGH can occur via deamidation of asparagine residues and oxidation of methionine residues in the solid or liquid states; the molecule can also form higher molecular weight aggregates with resultant instability (Becker et al., 1987). Preparation of growth hormone products using spray-and freeze-drying

techniques has previously been reported (Maa et al., 1998; Pikal et al., 1991).

The aim of this study was to prepare hGH powders using conventional and modified SEDS processes and to determine their integrity. The study was devised to provide further understanding of the SEDS process and thus aid in developing SF methods for the precipitation of hGH from aqueous solutions. The study included a range of classical bioanalytical and solid-state characterization methods such as ultraviolet (UV) spectroscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), reverse-phase high performance liquid chromatography (RP-HPLC), size exclusion chromatography (SEC), differential scanning calorimetry (DSC), X-ray powder diffraction (XRPD), scanning electron microscopy (SEM), and Karl Fischer techniques.

MATERIALS AND METHODS

Materials

An aqueous solution of hGH, stabilized by phosphate buffer, was supplied by Pfizer, Stockholm, Sweden. Before initiating the study, the source/bulk solution was filtered through 0.22 μm sterile filters (Millex-GV filter unit, Millipore Corp, Sundbyberg, Sweden) and the concentration was determined using spectrophotometry (45.3 mg/mL, Table 4). High purity (>99.9%) isopropanol, purchased from Merck, Germany, was used to enhance the extraction of water/solvent into SC-CO_2. Sucrose (purity >99.5%) was obtained from Sigma Aldrich, Germany. Quenched (amorphous) sucrose was obtained by melt quenching of raw material of sucrose in liquid nitrogen. Hydranal® composite 2 and Hydranal solvent, used in Karl Fischer titrations; were purchased from Sigma Aldrich Sweden AB, Stockholm. High purity CO_2 (99.9%) was obtained from AGA Gas AB, Sweden. MillQ water was used in most of the experimentation. All chemicals were used as supplied.

Preparation of hGH Powders

Precipitation of hGH Powders by Conventional SEDS

The description and schematics of the conventional SEDS equipment and operation procedure have been published elsewhere (Hanna & York, 1998). The aqueous solutions containing various proportions of isopropanol and protein solution (feed solutions) were fed through one inlet while SC-CO_2 was pumped through the other inlet of the two-component nozzle into a particle formation vessel. The diameter of the nozzle opening was 0.20 mm. The hGH particles were precipitated in the vessel as the water and solvent mixture was extracted into the SC-CO_2. Experiments were performed under various processing conditions (pressure 100–200 bars and temperature 40° and 50°C), and with various flow rates and solution compositions (CO_2/isopropanol and protein solution and sucrose in some cases). At the end of each experiment, the hGH powders were flushed with SC-CO_2 at the rate of 25 mL/min for 45–60 min to remove residual water and the solvent. All samples were weighed and stored for 1–5 months at −20°C, before the analysis. The powders precipitated using this method (T1-T6) are presented in Table 1.

TABLE 1 hGH Powders Precipitated Using the Conventional SEDS Process

Sample	hGH[a] (% w/v)	Isopropanol[a] (% v/v)	Sucrose[a] (% w/v)	P/T (Bars/°C)	%Yields[b]
T1	2	30	–	100/50	ND
T2	2	20	–	100/50	ND
T3	1	30	–	100/50	ND
T4	2	30	10	100/50	ND
T5	2	30	5	100/50	36 [1]
T6	2	30	5	100/40	25 [4]

The flow rates of the CO_2 and protein solutions were 25 and 0.1 mL/min, respectively, in all cases.

Note: ND = not determined; P/T = pressure/temperature.

[a]Concentrations in the feed solution.

[b]An approximate value calculated from the measured yield and the amount of material (hGH + sucrose) in the initial solution. Figures in brackets indicate standard deviations from three batches.

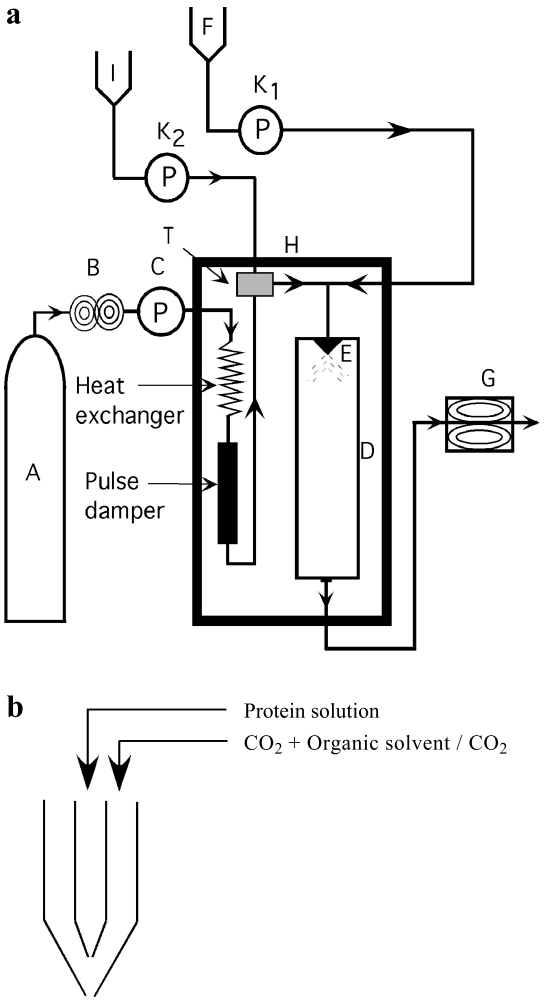

FIGURE 1 **a) Schematic Diagram of Modified SEDS Apparatus Showing the Specially Made T-piece. A: CO_2 Cylinder, B: Cooler, C: High Capacity Pump for Liquid CO_2, D: Particle Formation Vessel, E: Nozzle, F: Protein Feed Solution, G: Back Pressure Regulator, H: Oven, I: Solvent Modifier (Isopropanol), K_1: Feed Solution Pump, K_2: Solvent Pump, T: T-Piece; b) Cross-Section of the Nozzle Used in Both SEDS Methods.**

Precipitation of hGH Powders by Modified SEDS

Schematics of the apparatus and a cross-section of the nozzle used in this method (and in the standard method), are shown in Fig. 1. The modified SEDS method was basically the same as the standard process, except that the isopropanol was mixed with the SC-CO_2 via a specially made T-piece prior to entering the coaxial nozzle (which had a diameter of 0.35 mm). This creates a single-phase homogeneous mixture of SC-CO_2 and isopropanol (modified SC-CO_2). The water from the hGH solution was then extracted into the modified SC-CO_2, resulting in the formation of protein particles in the vessel.

A brief description of the process is as follows: The hGH feed solution (F) was pumped (K1) through the two-component coaxial nozzle. CO_2 was fed from the source (A) to a high-pressure pump (C) after liquification in the cooler (B). The pressure inside the particle formation vessel (D) was controlled by a back pressure regulator (G). The air-heated oven (H) controlled the temperature of the vessel. SC-CO_2 was modified in a specially made T-piece (T) by mixing with isopropanol (I), which was pumped by (K_2), prior to entering the coaxial nozzle (E). The water was extracted into the modified SC-CO_2 and protein particles were precipitated in the vessel. The pumps used for the protein solution and isopropanol (PU-980) and the back pressure regulator (880–81) were obtained from JASCO (Scandinavia AB, Molndal, Sweden). The T-piece was obtained from VALCO ACT (Schenkon, Switzerland) and the high pressure pump was obtained from Thar Design Inc. (Pittsburg, PA, USA).

Several experiments were performed under varying conditions, as in the standard experiments. In particular, experiments were performed under conditions (at high pressures and low temperatures) that did not allow precipitation of hGH with the standard SEDS procedure, to check for possible advantages. At the end of each experiment, the precipitated powders were flushed with modified SC-CO_2 at 25 mL/min for 10–12 min and later with SC-CO_2 for 60 min to remove residual water and isopropanol. All samples were weighed and stored for 1–5 months at −20°C, before the analysis. The powders precipitated using this method (V1–V5) are presented in Table 2.

Effect of Isopropanol on the Molecular Aggregation of hGH

In order to study the effect of isopropanol on the molecular aggregation behavior of hGH in the liquid and frozen states, various solutions of hGH in isopropanol were prepared. In some cases, sucrose was also added to study its stabilizing effect on isopropanol-induced hGH aggregation. Isopropanol was added to water for injection (WFI) and shaken until completely dissolved before slowly adding the hGH solution to a final hGH concentration of 2% w/v. In cases where sucrose was added, it was dissolved in WFI before the addition of isopropanol. The resultant solutions were mixed by gently rotating the

TABLE 2 hGH Powders Precipitated Using the Modified SEDS Process

Sample	hGH[a] (% w/v)	Isopropanol[a] (% v/v)	Sucrose[a] (% w/v)	P/T (Bars/°C)	%yields[b]
V1	1	5	–	100/50	38 [6]
V2	1	5	5	100/50	47 [5]
V3	1	–	5	100/50	61 [10]
V4	1	–	5	100/40	24 [4]
V5	2	–	5	100/50	54 [2]

The flow rates of CO_2, isopropanol, and hGH solutions were 25, 1, and 0.1 mL/min, respectively, in all cases.
Note: Figures in brackets indicate standard deviations from three batches.
P/T = pressure/temperature.
[a]Concentrations in the feed solution.
[b]An approximate value calculated from the measured yield and the amount of material in the initial solution.

container five times. Different solutions and frozen samples with their formulations are presented in Table 3. All samples were analyzed by SEC in order to assess the relative amounts of protein monomer, dimer, and oligomer.

Bioanalytical Characterization of hGH Powders

Bioanalytical characterizations in the study included determination of the concentration of hGH dissolved from the precipitated powders in water (using spectrophotometry), the presence of hGH in the remaining undissolved powder (SDS-PAGE), the chemical stability of hGH (RP-HPLC), and the relative content of soluble hGH monomers, dimers, and oligomers (SEC).

Measurement of Dissolved hGH from Powders

Spectrophotometry was used to determine the hGH content dissolved from the precipitated hGH powders.

TABLE 3 Concentrations of Isopropanol and Sucrose in Solutions and Frozen Samples Containing 2% w/v hGH

Samples	Isopropanol (% v/v)	Sucrose (% w/v)
Solutions		
S1	5	–
S2	10	–
S3	5	5
S4	5	10
S5	5	20
Frozen samples		
F1	5	–
F2	5	5

Specific amounts of protein powders were weighed and 1–2 mL of water was added. The samples were sonicated for 2 min to disperse agglomerates, shaken gently for 2 hours on a shake board, and centrifuged at 5000 rpm for 5–10 min. The supernatant was separated and filtered through a 0.22-μm sterile filter (Millex-GV filter unit, Millipore Corp, USA). The filtered supernatant was analyzed using SEC to determine the degree of protein aggregation in WFI. The concentration of soluble hGH was determined using a Lambda 2 UV/VIS spectrophotometer (Perkin–Elmer, Sweden) at 276 nm corrected for light scattering at 320 and 340 nm. A specific absorbance of 0.79 (1 mg/mL, 1 cm) was used. These solutions were used in later analyses as described in the following sections. The percentage of hGH recovered was calculated in relation to the theoretical amount of hGH in the powder, which is nothing but the amount of hGH in feed solution based on the assumption that there was no loss of protein (or sucrose) with the supercritical fluid.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This method was used to determine whether the undissolved solid that remained after 2 h of shaking and filtering contained protein, and also to investigate whether the protein was in monomer form or had been irreversibly aggregated. After separating the supernatants, which were used in SEC and spectrophotometry experiments (i.e., in measurement of dissolved hGH from powders section), the remaining solids were dissolved in 0.2 mL 10% sodium dodecyl sulfate (SDS) solution. Thus, the proteins were denatured by SDS to yield negatively charged complexes

of protein-SDS. The theoretical amount of undissolved hGH remaining in the solid was calculated from the total amount of protein originally added to WFI minus the amount of dissolved hGH in WFI after 2 hours' shaking (as measured by UV spectrophotometry; under measurement of dissolved hGH from powders section). In the SDS-PAGE experiments it was assumed that, if no protein had been lost during the SEDS process, the amount of hGH in the SDS solution was equal to the theoretical amount. These concentrations were then used to calculate the dilution factor prior to application of 1.3 mg/mL hGH solution to the SDS gel.

After diluting the protein-SDS solutions to a protein concentration of 1.3 mg/mL, the disulfide bonds in the protein were reduced with β-mercaptoethanol. The silver-stained SDS-PAGE method was performed in tris-glycine buffer according to Laemmli (1970). Each protein sample was loaded at 10 μg per lane. Separation was then obtained according to molecular size by electrophoresis in polyacryl amide gels (13.6% acrylamide) in the presence of SDS.

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

The effect of the processing on the chemical stability and degradation of hGH (i.e., oxidation and deamidation) was studied using RP-HPLC. The hGH solutions obtained above (i.e., in measurement of dissolved hGH from powders section) were analyzed using an HP 1090 (Hewlett Packard, Agilent technologies, Sweden) equipped with a polyvinyl alcohol-based Asahipak ODP50 column (250 × 4 mm, Hewlett Packard, RPC-134), equilibrated with 28% v/v 1-propanolol in 35.5 mM Tris-HCl buffer at pH 8.5 (mobile phase). The injection, volume of hGH (1.3 mg/mL was 30 μL and the flow rate was 0.5 mL/min at 55°C. Ultraviolet detection was carried out at 214 nm. The amounts were calculated by integrating the respective areas in the chromatogram.

Size Exclusion Chromatography (SEC)

The molecular size distribution of hGH was determined by SEC using HPLC 1100–3 (Hewlett Packard, Agilent technologies, Sweden). The supernatant (i.e., soluble) portions of the hGH solutions from above section (i.e., from measurement of dissolved hGH from powders) were diluted to 0.5–1.3 mg/mL and specific volumes of these solutions were injected into a Superdex 75 HR 10 × 30 column (with an inlet filter of 2 μm). The mobile phase consisted of 50 mM sodium dihydrogen phosphate (NaH_2PO_4), 50 mM disodium hydrogen phosphate (Na_2HPO_4), 100 mM sodium chloride, and 5.0% 2-propanol at pH 6.7, and was pumped at a flow rate of 0.8 mL/min. Protein concentration was measured at 214 nm. The concentration of hGH was analyzed by comparing the sample area response to the area response of reference standards. The proportions of monomer, dimer, and oligomer were determined relative to the total integrated areas under the elution curves.

Solid-State Characterization of hGH Powders Prepared by SEDS

Differential Scanning Calorimetry (DSC)

These experiments were performed on a DSC 220C (Seiko, Japan). Aluminum pans containing about 1.5–2 mg of hGH powders were hermetically sealed and two to three holes were made in the pan cover. The samples were ramped at 10°C before scanning from 10°C to 250°C at a rate of 10°C/min. Three measurements of each batch were made and reproducibility was verified by a second batch.

X-Ray Powder Diffraction (XRPD)

These experiments were performed on a conventional diffractometer (Diffractometer D5000, Siemens) operated with Cu K_α radiation (45 kV, 40 mA). The instrument was operated in step-scan mode at a rate of 0.2 in an angular range of 5–45°2θ. X-ray powder diffraction patterns of hGH powders were compared with those of crystalline (raw) and amorphous (quenched) sucrose.

Scanning Electron Microscope (SEM)

The size and morphology of hGH powders were examined using a scanning electron microscope (LEO

1530, Japan) at magnifications of 5–50 K. Small amounts of the powder were loaded on to the glue tape and the excess powder was blown away using an air stream of mild intensity. The particles were coated with gold-palladium (JFC-100, Ion Sputter, Jeol, Japan) in an argon atmosphere at room temperature before examination.

Residual Moisture Analysis

Residual moisture analysis was performed on a Karl Fischer automatic titrator (Metrohm 787 Titrino, Metrohm, Switzerland). Accurately weighed samples of hGH were transferred into the solvent vessel and titrated with the reagent. At the endpoint, the moisture content was automatically calculated from the titer volume and the volume of the reagent consumed. Each measurement was repeated three times and the moisture content was measured for three different batches of powders.

RESULTS AND DISCUSSION

Precipitation of hGH Powders by Conventional SEDS

Optimization of the Process

At the higher pressure (200 bar) and lower temperature (40°C (200/40), the precipitation process was incomplete with >20% v/v isopropanol and not possible at isopropanol concentrations <20% v/v. At 200 bars, it appears that the supersaturation in the water-rich phase was never achieved, resulting in no or only partial precipitation of the protein. In confirmation of this process, wet mass and few droplets of water were observed on the walls of the cylinder along with the pressure rise in the system. However, some hGH particles did precipitate when the pressure was decreased to 100 bar at 40°C (100/40) with an isopropanol content of >20% v/v. Further, at 100 bar and 50°C (100/50), complete precipitation of hGH was observed and small quantities were collected even at altered flow rates and concentrations of hGH. This may be the consequence of change in phase behavior with the reduction in pressure, as shown in Fig. 4. Therefore, optimal processing conditions were 100 bars pressure and 40–50°C at a protein solution flow rate of 0.05–0.1 mL/min and hGH concentrations of 1% and 2% w/v. Table 1 shows the results for samples obtained at a pressure of 100 bars and temperatures of 40° and 50°C. Flow rates of CO_2 and protein solution for these samples were 25 and 0.1 mL/min, respectively.

Effect of Isopropanol Concentration

Isopropanol concentrations of 10%, 20%, and 30% v/v were investigated (20% and 30% shown in Table 1; samples T1–T3). Results for 10% are not reported. It was not possible to quantify the amount of powder precipitated for some samples using 20–30% isopropanol because of low yields. For example, although the addition of 30% v/v isopropanol to the 2% hGH solution resulted in precipitation of the protein (T1), the powders were cohesive and the yields were too low to quantify. Precipitation of hGH was minimal with 20% isopropanol (the critical concentration essential for precipitation) and did not occur at all at concentrations below 20% v/v. At these low concentrations, a water-rich phase appeared to form. This phenomenon may have prevented precipitation, as might have occurred at the higher pressure of 200 bar. A similar result has been reported previously with lysozyme precipitation from water using ethanol-modified SF (Bustami et al., 2000). Decreasing the hGH concentration to 1% w/v (T3) had no significant effect on either the processing or precipitation yields (Table 1).

Effect of Sucrose

Sucrose was added in concentrations of 5% and 10% w/v (Table 1; samples T4–T6). The presence of sucrose reduced the required critical isopropanol concentration to as low as 10% v/v (data not shown) for precipitation. The addition of sucrose also allowed precipitation without intermittent pressure rises in the system, even at low concentrations (10–20% v/v) of isopropanol. It is assumed that the higher concentration of solutes in the system influenced the phase behavior/supersaturation profiles, resulting in precipitation at lower concentrations of cosolvent. There was a dramatic increase in the yield with 10% w/v sucrose (T4) compared with the samples that did not contain sucrose; however, the product was slightly damp, which thwarted efforts of quantification. Decreasing the

sucrose content to 5% w/v (T5) resulted in dry powder that was easier to collect (Table 1). However, different proportions of sucrose appeared to have no substantial influence on the precipitation phenomenon.

Effect of Temperature

The presence of 5% sucrose in the solution containing 30% v/v isopropanol allowed precipitation of hGH at 40°C (T6, Table 1), an event that was not possible without sucrose (results not shown). However, the yield of samples processed at 40°C was slightly lower than that at 50°C (T5, Table 1).

Precipitation of hGH Powders Using the Modified SEDS Apparatus

Process Optimization

A tendency for hGH to precipitate (i.e., few aggregates without water droplets) was observed at higher pressures and lower temperatures (200/40) than with the standard SEDS process, at certain flow rates. After extensive preliminary experimentation, pressure and temperature values of 100/50 and relative flow rates (CO_2/isopropanol/protein) of 25/1/0.1 were selected for further study because.of the ease of processing and product yields (Table 2).

Effect of Isopropanol

The addition of 5% v/v isopropanol in the solution of 1% w/v hGH (Table 2; sample V1) facilitated the precipitation, and the yields were higher compared to those that were precipitated from isopropanol solutions (Table 1; samples T1–T3) using the conventional SEDS process. The process was complete, without any interruption due to blocking of the nozzle. A similar outcome was observed when the flow rate of isopropanol was increased to 1.5 mL/min (data not shown). This may have been because the increased solvent ratios lead the equilibrium towards the homogeneous solvent-rich CO_2 phase, facilitating better extraction of water from the protein, but with an attendant risk of enhancing solvent effects on the protein.

Effect of Sucrose

The addition of 5% sucrose to 1% hGH and 5% isopropanol (sample V2) increased the product yield and further facilitated the precipitation process. This result was consistent with the conventional SEDS process. Surprisingly, the presence of 5% w/v sucrose in the protein solution (without isopropanol) also facilitated hGH precipitation using modified SC-CO_2, resulting in a yield as high as 61% (Table 2; sample V3). The precipitation process was uninterruptible and complete. In this sample, hGH was precipitated from a pure aqueous solution, dramatically reducing the solvent effects on the protein using the modified SEDS process.

Effect of Temperature

Decreasing the temperature to 40°C had a dramatic effect on the precipitation of the protein from the

TABLE 4 **Amount of hGH Dissolved from Precipitated hGH Powders**

Samples	Soluble hGH[a] (mg/mL)	Theoretical hGH loading[b] (mg/mL)	% Recovery	n
hGH[b]	45.3	–	–	1
T1–T4	ND	ND	ND	1
T5	3.9 (0.4)	8.6 (0.2)	45.3 (4.8)	4
T6	3.2	8.6	37.2	1
V1	0.1 (0.0)	14.3 (0.3)	0.9 (0.0)	3
V2	2.9 (0.6)	4.9 (0.3)	63.2 (13.2)	5
V3	3.7 (0.6)	5.2 (0.3)	71.2 (6.8)	3
V4	1.2 (0.7)	2.7 (0.5)	51.8 (31.8)	4
V5	1.9 (0.8)	8.8 (0.6)	9.7 (1.8)	4

Mean values with standard deviations in brackets.
Note: n = number of batches; ND = not determined (low concentrations of hGH).
%hGH dissolved or recovery = (amount dissolved/amount in the powder) × 100.
[a]Measured experimentally using spectrophotometry.
[b]Bulk solution.

solution containing 5% w/v sucrose and 1% w/v hGH (Table 2; sample V4). The extraction was partial, with few droplets of water inside the cylinder, and the production yield, although quantifiable, was lower than at 50°C. At 40°C, as in the case of V4, the mixture at equilibrium may exist in a water-solvent rich phase, resulting in the poor and incomplete precipitation of protein with more interaction with the solvent. While at 50°C, as in the case of V3, the mixture probably exists in the CO_2-rich homogeneous phase, resulting in the rapid, complete precipitation of the protein, possibly with less interaction with the solvent. There were no substantial differences in precipitation behavior due to the increase in the concentration of hGH from 1% (V3) to 2% w/v (V5).

Bioanalytical Characterization

The results of the various bioanalytical investigations are presented in Tables 4–6.

hGH Bulk, Solutions, and Frozen Samples

The bulk hGH solution contained 45.3 mg/mL hGH as measured by spectrophotometry (Table 4). The RP-HPLC analysis of bulk solution showed that the amount of native hGH was 98%, and that 99.8% of hGH was in monomeric form as shown by SEC (Tables 5 and 6). Even solutions of hGH containing isopropanol that had been frozen and then thawed showed no substantial chemical degradation (Table 5; F1) and were not influenced by the addition of 5% sucrose (F2). The SEC analysis of solutions containing only 2% hGH and 5–10% isopropanol (S1 and S2) or containing hGH plus 5% isopropanol and 5–20% sucrose (S3–S5) showed that about 99.7% was still in monomer form, almost the same proportion as that in the bulk solution (Table 6). Solutions of hGH containing isopropanol that had been frozen (Table 3; F1 and F2) showed significant aggregation of the protein even in the presence of sucrose (Table 6).

hGH Powders Precipitated from Solutions Containing Isopropanol

Very small amounts of hGH were dissolved from precipitated powders T1–T3 and V1 (Table 4), all of which contained isopropanol but not sucrose. In fact, the dissolution characteristics of powders precipitated using modified SEDS were no better than those prepared using the conventional process. The hGH concentrations in the solutions obtained from these

TABLE 5 Degradation of hGH, as Measured by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

Samples	M(%)	A(%)	B(%)	n
hGH[a]	98	0.3	1.7	1
Frozen samples				
F1	97.3	0.3	2.9	1
F2	97.5	0.3	2.9	1
hGH samples from SEDS methods				
T1–T4	ND	ND	ND	1
T5	97.2(0.5)	0.4(0.1)	1.9(0.3)	4
T6	96.6	0.6	2.4	1
V1	ND	ND	ND	1
V2	92.2(5.6)	2.3(0.5)	4.7(4.1)	5
V3	92.8(5.0)	2.9(2.2)	5.7(6.0)	4
V4	93.2(2.5)	1.7(0.4)	4.4(1.8)	4
V5	94.6(2.3)	1.1(0.2)	3.7(1.8)	3

Mean values with standard deviations in brackets.
Note: M = rhGH and (trisulphide Cys182-Cys189)-rhGH (i.e., native/intact hGH).
A = (Met(O)125)-rhGH and (des-Phe1, des-Pro2)-rhGH.
B = (Met(O)14)-rhGH, (des-Phe1)-rhGH, clipped 142/143)-rhGH and deamidated rhGH.
D = (Nle14)-rhGH and (Nle125)-rhGH $\leq$ 0.2% for all the samples (not in the table).
n = number of batches; ND = not determined (low concentrations of hGH).
[a]Bulk solution.

TABLE 6 Size Exclusion Chromatography (SEC) Analysis of Various hGH Samples

Sample	Monomer (%)	Dimer (%)	Oligomer (%)	n
hGH[a]	99.8	0.2	<0.1	1
Solutions of hGH				
S1–S5	99.7(0.1)	0.2–0.3	<0.1	1
Frozen samples				
F1	50.1	10.4	39.6	1
F2	55.0	16.7	28.3	1
hGH samples from SEDS methods				
T1–T3	ND	ND	ND	1
T4	76.9	22.2	0.9	1
T5	76.9(1.0)	18.5(3.0)	4.6(2.9)	4
T6	73.4	21.2	5.4	1
V1	ND	ND	ND	3
V2	88.6(6.1)	7.0(3.6)	3.4(2.5)	5
V3	91.6(6.1)	7.0(4.6)	1.5(1.5)	5
V4	52.1(24.8)	21.8(10.1)	26.1(15.2)	4
V5	71.8(12.8)	14.7(5.9)	13.6(7.0)	4

Mean values with standard deviations for various batches in brackets.
Note: ND = not determined (amounts of hGH too low); n = number of batches.
[a]Bulk solution.

powders were too low to allow analysis by RP-HPLC and SEC (Tables 5 and 6).

hGH Powders Precipitated from Solutions Containing Sucrose

The amount of hGH dissolved from the precipitated powders increased with the addition of 5% sucrose to the feed solutions (Table 4). With 2% hGH solution in the conventional SEDS process (i.e., a ratio of 2:5 for hGH:sucrose), 37–45% of the protein was dissolved and measured using spectrophotometry. However, with 1% hGH solution in the modified SEDS process (ratio 1:5), 52–71% of the protein was dissolved (Table 4). This result clearly demonstrates an advantage of the modified SEDS process. Though the percent recovery values presented in Table 4 were assumed to be underestimated, it was clear that the highest recovery of 71% was observed for sample V3 that was precipitated using the modified SEDS process. The presence of isopropanol in sample V2 and the drop in the processing temperature in sample V4 had only a minor influence on the proportion of protein recovered from the powders (Table 4). However, the percent recovery of hGH from V5, which contained a higher protein-to-sucrose ratio (2:5) than the other samples (and the same ratio as that in the samples from the conventional process) was exceptionally low (Table 4). These results suggest that there were no deleterious effects of the processing/solvent on the protein in the presence of sucrose and no significant aggregation. In conclusion, it can be speculated that the sucrose limits protein aggregation during precipitation and assists dissolution by maintaining a high protein surface area and/or by diluting the protein in an amorphous hydrophilic matrix.

The SDS-PAGE analysis of the insoluble solids obtained from powders T4–T6 and V2, V3, and V5 showed that 99% of undissolved hGH was in non-covalent aggregates and/or reducible covalent aggregates. The SDS-PAGE estimations of the amounts of hGH in the insoluble solids were in agreement with the theoretical amounts calculated from the amount dissolved (using the UV concentration) and the initial amount of hGH in the feed solution.

From Table 5, RP-HPLC data for powders containing sucrose indicated that the chemical degradation of hGH was low i.e., 4–6% to that of bulk solution. Indeed, the method of precipitation, the protein:sucrose concentration ratios, and the temperature did not have any considerable effect on the stability of the protein. The product that was least chemically degraded after processing was T5. This product was prepared from a solution containing 30% isopropanol, an addition that is commonly known to decrease hydrolytical reactions in aqueous solutions.

The SEC analysis of the powders also indicated that the presence of sucrose reduced the risk of aggregation

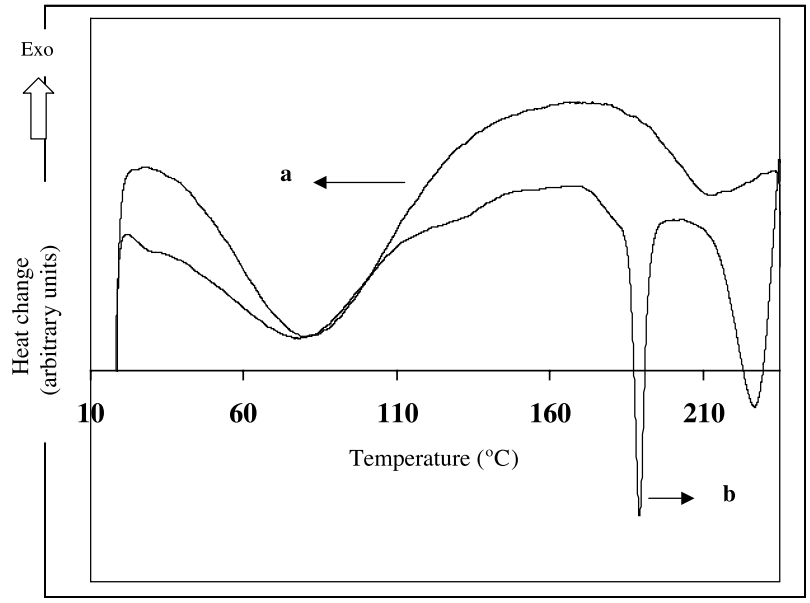

FIGURE 2 **DSC Thermograms of hGH Powder a) Precipitated from Solutions Containing Isopropanol and No Sucrose (V1) and b) Precipitated from Solutions Containing Sucrose and No Isopropanol (V3).**

during precipitation (Table 6). This could be due to the protective nature of the sucrose against solvent/process stresses in the SEDS (Fransson et al., 1997). The sucrose molecules may have provided stability against aggregation by diluting the protein and thus reducing intermolecular reactions. In powders T4–T6 from conventional SEDS, the monomer content was 73–77%. The hGH:sucrose ratio (e.g., samples T4 and T5) and the processing temperature (sample T6) had no substantial influence on the monomer content (Table 6). The monomer content in samples V2–V5 from modified SEDS was 52–92 (Table 6). In sample V2, which contained 5% each of both sucrose and isopropanol, the proportion of monomer particles was high, indicating minimized detrimental effects of the solvent on the physical stability of hGH. In support of this hypothesis, the monomer content was even higher in sample V3, which contained only sucrose and no isopropanol (Table 6). The higher monomer content in samples V2 and V3 than in samples T4 and T5 indicates an advantage associated with the modified SEDS process in that exposure of hGH to the solvents is minimized. Interestingly, a reduction in temperature to 40°C, even

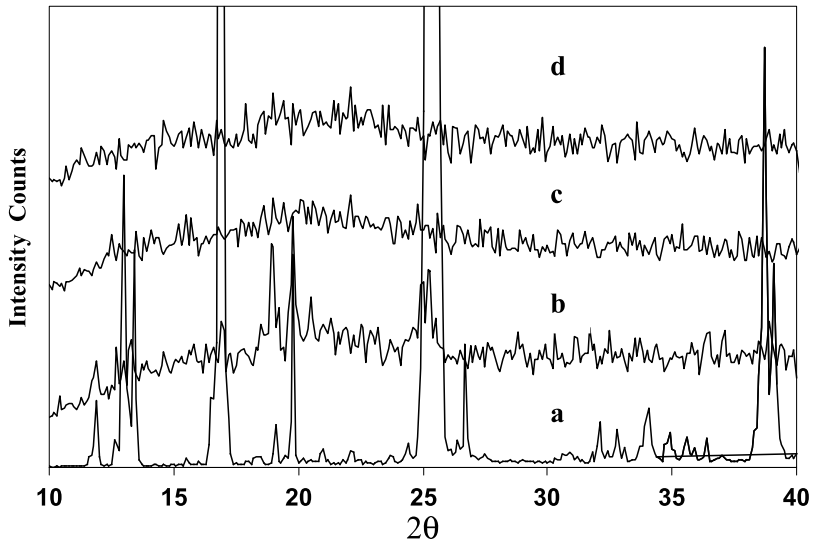

FIGURE 3 **XRPD Patterns of a) Crystalline Sucrose; b) Quenched Sucrose; c) T4, Containing 2% hGH, 30% Isopropanol, and 10% Sucrose (Conventional SEDS); d) V3, Containing 1% hGH and 5% Sucrose (Modified SEDS).**

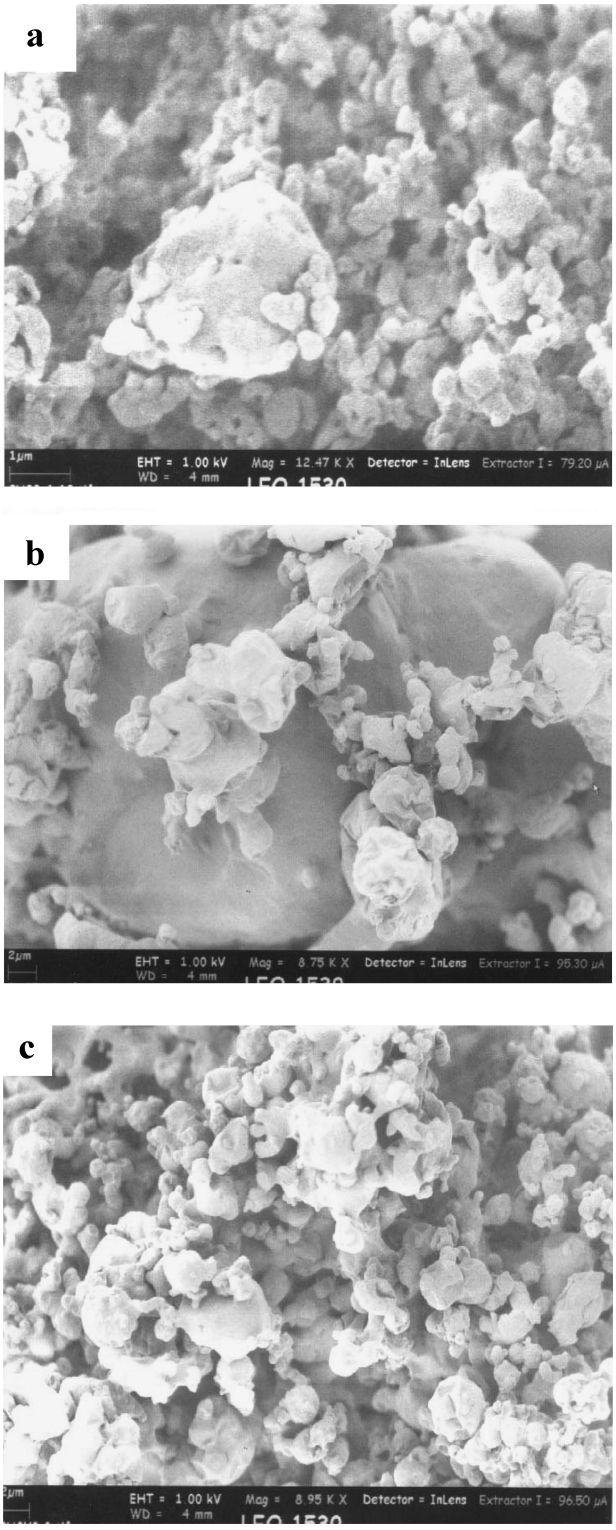

FIGURE 4 **SEM Pictures of a) hGH Powder V1, Precipitated from Solution Containing 1% hGH and 5% Isopropanol; b) T4, Containing 2% hGH, 30% Isopropanol, and 10% Sucrose; c) V3, Containing 1% hGH and 5% Sucrose.**

in the presence of sucrose, had a notable influence on the hGH structure, resulting in about 50% of aggregated product (sample V4, Table 6). This could be the result of precipitation of the protein from the solvent-rich phase, as discussed earlier. Increasing the protein:sucrose ratio to 2:5 in the feed solution (V5) resulted in 20% higher aggregation than seen with sample V3 (ratio 1:5). This supports the suggestion that sucrose offers protection by dilution during the precipitation process, as discussed above.

Solid-State Characterization

Data on the thermal behavior, crystal structure, morphology, and moisture content are presented in Figs. 2–4 and Table 7.

Crystalline and Amorphous Sucrose

The DSC analysis indicated that crystalline sucrose melted at around 189°C, with a huge enthalpy of fusion, while no clear melting endotherm was seen for quenched sucrose, as is typical of amorphous materials. The XRPD patterns of crystalline and quenched sucrose, used as crystalline and amorphous reference standards, are shown in Fig. 3. The X-ray diffraction profile of crystalline sucrose showed intense characteristic peaks at 2θ angles of 13, 17, 19.5, 25, and 39 (Fig. 3a). The characteristic peaks of the crystalline phase in the quenched sucrose sample were diminished, confirming the predominantly amorphous phase of this sample (Fig. 3b).

hGH Powders Precipitated from Solutions Containing only Isopropanol

The DSC analysis of powders precipitated from solutions (both methods) containing only isopropanol (i.e., without sucrose; samples T1–T3 and V1) showed a substantial endotherm at around 80°C and a smaller one at 214±1°C. The initial (large endotherm) transition could be attributed to the evaporation of residual moisture and the smaller transition may have been the result of the native protein structure unfolding due to high temperature denaturation (Fig. 2). Karl Fischer analysis confirmed higher residual moisture content in sample V1 (Table 7).

The precipitated powders, irrespective of method of preparation, showed diffuse peaks in the XRPD

TABLE 7 Percentage Residual Moisture Content in Precipitated hGH Powders (Karl Fischer Analysis)

	% Residual moisture content			
	Within one batch[a]		Among different batches[b]	
Sample	Mean	SD	Mean	SD
T1–T4	NA	NA	NA	NA
T5	9.66	1.37	8.74	1.73
V1	8.30	0.42	NA	NA
V2	8.50	0.50	10.42	3.55
V3	5.19	0.83	6.64	1.87
V4	NA	NA	NA	NA
V5	6.27	0.58	NA	NA

Note: NA = not analyzed.
SD = standard deviation ($n = 3$).
[a]Provides variability in the analysis method.
[b]Provides variability among powdered batches from SEDS.

analyses, indicating that they were amorphous in nature (e.g., sample V1, not shown in figure but similar to Fig. 3c). As exemplified by the SEM picture of sample V1 (Fig. 4a), particles of the powders, regardless of process set-up or other variables, were generally partially spherical and aggregated, with smaller particles on the surface of the larger ones.

hGH Powders Precipitated from Solutions Containing Sucrose

The DSC profiles of powders containing sucrose (samples T4–T6 and V2–V5) showed an endotherm with an onset at around 30°C followed by a sharp endothermic transition at around 189°C and a final endotherm with a peak at 224±2°C. The initial transition may be the result of evaporation of residual moisture, and the transition at 189°C was from the melting of sucrose (Fig. 2). It was observed that the presence of sucrose in the formulations delayed the third endothermic event, probably as a result of the stabilizing effects of sucrose (data not shown). Similar thermal behavior was observed for powders precipitated from both processes and at different temperatures and protein:sucrose concentration ratios. Though it was not clear from the evaporation endotherms in the DSC thermograms, powders containing 5% sucrose prepared under optimum conditions had lower moisture content than other samples (see Fig. 2 and samples V1 vs. V3, V5 in Table 7). The XRPD patterns indicated that these powders were also amorphous, regardless of process and formulation variables (Figs. 3c and 3d). However, an exothermic peak corresponding to the recrystallization of the amorphous phase was not seen in the DSC profile. It may be that substantial recrystallization of amorphous carbohydrates is delayed or prevented in the presence of globular proteins such as hGH (Costantino et al., 1997; Mosharraf, 2003; Souillac et al., 2002). As seen from SEM pictures of T4 and V3 (Figs. 4b and 4c), powders containing sucrose were similar in morphology or shape to those without sucrose (Fig. 4a), irrespective of process and formulation variables. However, particles of powders precipitated under optimum conditions using the modified SEDS process (e.g., sample V3) were smoother, with a narrower and more uniform particle size distribution than other samples (Fig. 4c), i.e., approx. 1–6 μm. The amorphous nature of the particles is also supported by their spherical appearance in these SEM pictures (Fig. 4).

CONCLUSIONS

In this study, optimal conditions (pressure of 100 bars and temperature 40–50°C) for the precipitation of hGH powders using conventional and modified SEDS were identified. It was found that hGH precipitates from either the solvent or the CO_2-rich phase, depending on the mixture composition. Modifying the SC-CO_2 by the addition of isopropanol prior to dispersion initiated the precipitation effectively, even with low quantities of isopropanol in the solution. The addition of sucrose (without isopropanol) to the aqueous solutions of the protein also facilitated precipitation; thus, in the modified

SEDS process, hGH was precipitated entirely from aqueous solutions.

The amount of hGH dissolved from the precipitated powders, which was extremely low in the presence of isopropanol alone, was dramatically increased by the addition of sucrose. Reverse-phase HPLC confirmed more than 92% intact hGH. The soluble hGH monomer content was 52–92%, as shown by the SEC study, in the presence of sucrose. A higher hGH monomer content was obtained for some powders obtained using the modified SEDS method; however, the temperature and protein:sucrose concentration ratio had a strong influence on this outcome. Powders were amorphous and consisted of fused aggregates of partially spherical particles with 5–10% residual moisture content. The solid-state characteristics and shape/morphology were independent of the method of processing, the presence of sucrose, and the processing conditions.

In summary, an optimal formulation of hGH in this study was obtained (highest yield 61%) at 100 bars pressure and 50°C when precipitated from aqueous solution containing 1% hGH and 5% sucrose using the modified SEDS method. Powders were amorphous, with particles approximately 1–6 μm in diameter and containing 5% residual moisture. This formulation also showed the highest proportion (71%) of recovered (dissolved) hGH. The protein was stable against chemical degradation during processing and was mainly (92%) recovered as the monomer. Clearly, further optimization of the process is necessary before obtaining a product that meets the requirements of a pharmaceutical product. The fraction of insoluble aggregates and moisture contents must be reduced. This feasibility study has added to the understanding of the SF process for precipitating proteins and offers potential for the development of an alternative process for the preparation of stable powders of hGH.

REFERENCES

Allison, S. D., Randolph, T. W., Manning, M. C., Middleton, K., Davis, A., & Carpenter, J. F. (1998). Effects of drying methods and additives on structure and function of actin: mechanisms of dehydration-induced damage and its inhibition. *Archives of Biochemistry and Biophysics, 358*, 171–181.

Arakawa, T., Prestrelski, S. J., Kenney, W. C., & Carpenter, J. F. (1993). Factors affecting short-term and long-term stabilities of proteins. *Advanced Drug Delivery Reviews, 10*, 1–28.

Becker, G. W., Bowsher, R. R, Bromer, W. W., MAcKEllar, W. C., Poor, M. L., & Tackitt, P. M., et al. (1987). Chemical, physical and biological characterisation of a dimeric form of biosynthetic human growth hormone. *Biotechnology and Applied Biochemistry, 9*, 478–487.

Bustami, R. T., Chan, H. K., Dehghani, F., & Foster, N. R. (2000). Generation of microparticles of proteins for aerosol delivery using high pressure modified carbon dioxide. *Pharmaceutical Research, 17*, 1360–1366.

Costantino, H. R., Curley, J. G., & Hsu, C. C. (1997). Determining the water sorption monolayer of lyophilized pharmaceutical proteins. *Journal of Pharmaceutical Sciences, 86*, 1390–1393.

Debenedetti, P. G., Tom, J. W., & Yeo, S.-D. (1993). Supercritical fluids: a new medium for the formation of particles of biomedical interest. In: *Proceedings of 20th int. symp. on controlled release bioactive materials*. Controlled Release Study 141–142.

Dillow, A. K., Dehghani, F., Hrkach, J. S., Foster, N. R., & Langer, R. (1999). Bacterial inactivation by using near-and supercritical carbon dioxide. *Proceedings of the National Academy of Sciences of the United States of America, USA 96*, 10344–10348.

Fransson, J., Hallen, D., & Florin-Robertsson, E. (1997). Solvent effects on the solubility and physical stability of human insulin-like growth factor I. *Pharmaceutical Research, 14*, 606–612.

Hanna, M., & York, P. Method and Apparatus for the Formulation of Particles. Eur. Patent 9313642.2, 1998.

Jackson, M., & Mantsch, H. H. (1991). Beware of protein in DMSO. *Biochemical and Biophysical Acta, 1078*, 231–235.

Krukonis, V. (1984). *Supercritical fluid nucleation of difficult-to-comminute solids*. Proceedings of AIChE meeting: San Francisco, USA.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Loth, H., & Hemgesberg, E. (1986). Properties and dissolution of drugs micronised by crystallization from supercritical gases. *International Journal of Pharmaceutics, 32*, 265–267.

Maa, Y. F., Nguyen, P. A., & Hsu, S. W. (1998). Spray-drying of air-liquid interface sensitive recombinant human growth hormone. *Journal of Pharmaceutical Sciences, 87*, 152–159.

Manning, M. C., Patel, K., & Borchardt, R. T. (1989). Stability of protein pharmaceuticals. *Pharmaceutical Research, 6*, 903–918.

Mosharraf, M. (2003). Assessment of degree of amorphicity (disorder) of Lyophilized formulations of growth hormone using isothermal microcalorimetry. *Drug Development and Industrial Pharmacy. In Press.*

Moshashaee, S., Bisrat, M., Forbes, R. T., Nyqvist, H., & York, P. (2000). Supercritical fluid processing of proteins I: Lysozyme precipitation from organic solution. *European Journal of Pharmaceutical Sciences, 11*, 239–245.

Moshashaee, S., Bisrat, M., Forbes, R. T., Quinn, E. A., Nyqvist, H., & York, P. (2003). Supercritical fluid processing of proteins: lysozyme precipitation from aqueous solution. *Journal of Pharmacy and Pharmacology, 55*, 185–192.

Nesta, D. P., Elliott, J. S., & Warr, J. P. (2000). Supercritical fluid precipitation of recombinant human immunoglobulin from aqueous solutions. *Biotechnology and Bioengineering, 67*, 457–464.

Palakodaty, S., York, P., & Pritchardt, J. (1998). Supercritical fluid processing of materials from aqueous solutions: the application of SEDS to lactose as a model substance. *Pharmaceutical Research, 15*, 1835–1843.

Pikal, M. J. (1994). Freeze-drying of proteins: process, formulation, and stability. In: Pikal, M. J. *Formulation and delivery of proteins and peptides*. vol. 567. ACS Symposium Series. 120–133.

Pikal, M. J., Dellerman, K. M., Roy, M. L., & Riggin, R. M. (1991). The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharmaceutical Research, 8*, 427–436.

Randolph, T. W., Randolph, A. D., Mebes, M., & Yeung, S. (1993). Sub-micrometer-sized biodegradable particles of poly(L-lactic acid) via the gas antisolvent spray precipitation process. *Biotechnology Progress, 9*, 429–435.

Sellers, S. P., Clark, G. S., Sievers, R. E., & Carpenter, J. F. (2001). Dry powders of stable protein formulations from aqueous solutions prepared using supercritical CO(2)-assisted aerosolization. *Journal of Pharmaceutical Sciences, 90*, 785–797.

Souillac, P. O., Costantino, H. R., Middaugh, C. R., & Rytting, J. H. (2002). Investigation of protein/carbohydrate interactions in the dried state. 1. Calorimetric studies. *Journal of Pharmaceutical Sciences, 91*, 206–215.

Winters, M. A., Knutson, B. L., Debenedetti, P. G., Sparks, H. G., Przybycien, T. M., & Stevenson, C. L., et al. (1996). Precipitation of proteins in supercritical carbon dioxide. *Journal of Pharmaceutical Sciences, 85*, 586–594.

Yeo, S.-D., Lim, G.-B., Debenedetti, P. G., & Bernstein, H. (1993). Formation of microparticulate protein powders using a supercritical fluid antisolvent. *Biotechnology and Bioengineering, 41*, 341–346.

York, P. (1995). A novel approach for controlled particle formation using supercritical fluids. *Pharmaceutical Research, 12*, S141.