Poly(Lactide-Co-Glycolide) Microsphere Formulations of Darbepoetin Alfa: Spray Drying Is an Alternative to Encapsulation by Spray-Freeze Drying

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Purpose. The purpose of this work was to evaluate spray-freeze drying and spray drying processes for encapsulation of darbepoetin alfa (NESP, Aranesp®).

Methods. Darbepoetin alfa was encapsulated in poly(lactide-co-glycolide) by spray-freeze drying and by spray drying. Integrity was evaluated by size-exclusion chromatography and Western blot. Physical properties and *in vitro* release kinetics were characterized. Pharmacokinetics and pharmacodynamics were evaluated in nude rats.

Results. Microspheres produced by spray drying were larger than those produced by spray-freeze drying (69 μm vs. 29 μm). Postencapsulation integrity was excellent for both processes, with <2% dimer by size-exclusion chromatography. *In vitro* release profiles were similar, with low burst (<25%) and low cumulative protein recovery at 4weeks (≤30%), after which time covalent dimer (≤6.5%) and high molecular weight aggregates (≤2.3%) were recovered by denaturing extraction. After a single injection, darbepoetin alfa was detected in serum through 4 weeks for all microsphere formulations tested *in vivo*, although relative bioavailability was higher for spray-freeze drying (28%) compared with spray drying (21%; $p = 0.08$) as were yields (73–82% vs. 34–57%, respectively). For both processes hemoglobin was elevated for 7 weeks, over twice as long as unencapsulated drug.

Conclusions. Spray drying, conducted at pilot scale with commercial equipment, is comparable to spray-freeze drying for encapsulation of darbepoetin alfa.

KEY WORDS: protein stability; microencapsulation; controlled release; novel erythropoiesis-stimulating protein (NESP); poly(lactide-co-glycolide) (PLGA).

INTRODUCTION

A major technical hurdle in the development of protein microsphere formulations (1,2) is the maintenance of integrity through encapsulation, where processing requirements can include temperature extremes and organic solvent exposure. Spray-freeze drying, a multistep cryogenic process (Fig. 1) developed specifically for protein therapeutics (3), has seen multiple applications at a bench-top scale (4). The scaled process is used in the commercial manufacture of Nutropin Depot™, a marketed controlled release formulation of human growth hormone (5).

Spray drying is an alternative process whereby microspheres are formed directly from an atomized spray (Fig. 1), avoiding cryogenic conditions and the lengthy ethanol extraction step required by spray-freeze drying. Spray drying has been used to encapsulate numerous small molecule drugs (6,7), and in the commercial manufacture of Parlodel® (8), a poly(lactide-co-glyocolide) (PLGA) formulation of bromocriptine. Commercial spray dryers are used widely in the pharmaceutical industry for a variety of applications (9). Bovine serum albumin (10–12) and glutathione *S*-transferase (13) were encapsulated as model proteins and/or antigens in bioerodible polyesters by spray drying emulsions. The latter approach resulted in significant covalent aggregation of recombinant human erythropoietin (rHuEPO; Ref. 14). Proteins best withstand stresses of organic solvent exposure and heat when encapsulated as solid dispersions (4,15), conditions used with bovine serum albumin (16), and bovine somatotropin (17). High burst and little or no sustained drug release were observed. The bench-top spray dryers used in these studies produce small (0.5–5 μm) microspheres; incomplete encapsulation of micron-sized drug particles can result. Submicron protein particles are made only with great difficulty, rendering bench-top spray dryers unattractive for encapsulation of solid dispersions.

Erythropoietin is a sialoglycoprotein hormone regulating the body's red blood cell mass (18). Darbepoetin alfa (novel erythropoiesis-stimulating protein [NESP], Aranesp®; Ref. 19) contains five N-linked glycosylation sites, two more than rHuEPO. Compared to rHuEPO, darbepoetin alfa exhibits an increased terminal half-life allowing for reduced dosing frequency, resulting in less disruption to patients' lives. A polymeric microsphere formulation of darbepoetin alfa may offer the additional advantage of controllably extending duration of action from a single darbepoetin alfa injection. The objective of the present study was to evaluate spray drying as an alternative to spray-freeze drying for the encapsulation of darbepoetin alfa. Spray drying was conducted at a pilot scale under conditions expected to result in microspheres large enough to ensure complete encapsulation of ≈2 μm darbepoetin alfa particles (20). The impact of process on protein integrity and microsphere properties, including *in vitro* release kinetics and *in vivo* performance, was evaluated.

MATERIALS AND METHODS

Materials

Darbepoetin alfa was from Amgen (Thousand Oaks, CA, USA). α,α-Trehalose dihydrate (high purity, low endotoxin) was from Pfanstiehl (Waukegan, IL, USA). Poly(lactide-co-glycolide) 50:50 (RG504H, inherent viscosity 0.49 dL/g in chloroform) was from Boehringer Ingelheim (Ingelheim, Germany). Dichloromethane (USP-NF grade) was from J.T. Baker (Phillipsburg, NJ, USA) and ethanol (USP grade) from Aaper (Shelbyville, KN, USA). High-purity (99.9%) USP carbon dioxide was from Praxair (Torrance, CA, USA). Bioburden assay reagents were from BBL, Becton Dickinson Micro System (Cockeysville, MD, USA). All other chemicals

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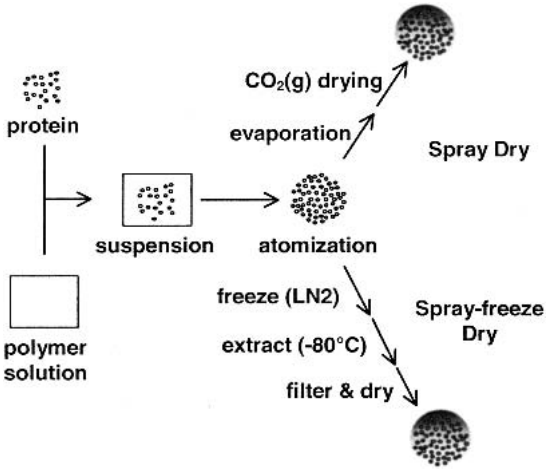

Fig. 1. Schematic of spray-freeze dry and spray dry encapsulation processes. A suspension of solid protein particles in polymer solution is atomized to form nascent microspheres in each process. In spray-freeze drying, the atomized spray is frozen in liquid nitrogen (LN2), followed by extraction of the polymer solvent in cold (–80°C or –40°C) ethanol for hours to days. The microspheres are collected by filtration and dried under vacuum. In spray drying, the polymer solvent is removed by evaporation. In the present work spray dried microspheres were further dried using carbon dioxide gas (22).

were of analytical grade or purer and were from commercial suppliers.

Methods

Encapsulation by Spray-Freeze Drying

Darbepoetin alfa powder, with a nominal composition of 45% darbepoetin alfa, 25% trehalose, and 30% sodium phosphate (wt%), was prepared by spray drying an aqueous solution as described (20) and encapsulated by a spray-freeze drying process similar to that described (21). Darbepoetin alfa powder (12.5 mg, corresponding to a 1-g microsphere lot size) was combined with 15.1 mL of a 6.5% (w/v) solution of PLGA in methylene chloride and vortexed thoroughly. The ambient suspension was atomized in two equal portions corresponding to 0.5-g sublots into frozen ethanol (200 mL) with a liquid nitrogen overlay (350 mL) using a 6-mm diameter, 20-kHz ultrasonic atomization probe (Sonics and Materials, Danbury, CT, USA) at 2.5 W, with a feed rate of 0.5 mL/min. The mixture was cured at –80°C for ≥72 h. Microspheres were collected by filtration and dried by lyophilization. The two sublots were combined, sieved (125 μm, 115 mesh stainless steel), and stored at –40°C.

Encapsulation by Spray Drying

Darbepoetin alfa powder, prepared at pilot scale as described (20), was encapsulated by spray drying using a Niro Mobile Minor® equipped with a 0.11-m^3 drying chamber extension, and a 25-kHz ultrasonic atomization nozzle (Sono-Tek, Milton, NY, USA) at 1.3 watts. Industrial grade nitrogen was used for atomization and drying as described (20). For a 10-g microsphere lot, darbepoetin alfa powder (125 mg) was combined with 114 ml of an 8.7% solution of PLGA in methylene chloride. The mixture was bath sonicated (30 s; Branson model 2210) and pumped (Harvard Apparatus syringe pump; Holliston, MA, USA) from a gas tight syringe (SGE, Austin, TX, USA) through 1/8" i.d. Viton tubing. Process conditions were as follows: feed temperature, ambient; feed flow rate, 12 ml/min.; drying gas flow rate, 1.2 lb/min (440 SLPM); inlet temperature, 55°C. The resulting outlet temperature was approximately 28°C. After spraying, unheated drying gas flow was maintained for 60 min, during which time inlet and outlet temperatures dropped below 25°C. Microspheres from the chamber collection point were dried further by carbon dioxide gas in an extraction chamber at regulated pressure as described (22). To avoid particle agglomeration pressure was increased from atmospheric to 100 psi at the initiation of drying, followed by a subsequent increase to 300 psi at 24 h. Drying continued at 300 psi for a total drying time of 42 h. Dried microspheres were sieved as above and stored at –80°C.

Physical Characterization

Particle size was determined by laser light scattering using a Malvern Mastersizer X. Microspheres were suspended in water containing 0.1% polysorbate 80 and sonicated prior to analysis. Span, a measure of polydispersity, was defined as $(D_{90} - D_{10})/D_{50}$ where D_i is the volume diameter at i cumulative volume percent. Microsphere morphology was characterized by scanning electron microscopy (SEM) using an AMR1000. Samples were mounted on a carbon adhesive tab and sputter coated (10 – 15 nm) with gold palladium (60/40 alloy). Bulk density was measured as described (23). True density was determined by helium pycnometry using an AccuPyc 1330 (Micromeritics, Norcross, GA, USA). Triplicate samples (30 mg) were analyzed at room temperature, with an equilibration rate of 0.005 psig/min and expansion volume of 0.691 ml. Residual methylene chloride and ethanol were analyzed in duplicate by gas chromatography on a Hewlett Packard 6890 equipped with a headspace analyzer (HP7694) and a CP-HT PoraPak Q 25 m column (Chrompack, The Netherlands) at 140°C. The carrier gas was helium (9.3 psi, 10 mL/min). Detection was by flame ionization, with a hydrogen flow of 40 mL/min, helium make-up gas flow of 35 mL/min, and air flow of 450 mL/min. Microspheres were dissolved in 0.5 M sodium hydroxide prior to gas chromatography analysis.

Characterization of Protein Integrity

Darbepoetin alfa was recovered from microspheres by extraction. Acetonitrile (1.0 mL) was added to 20–40 mg of microspheres, and the mixture vortexed until the polymer dissolved. After centrifugation (17,500 g), the supernatant was removed, and the pellet washed three times. The pellet was vacuum dried and reconstituted in 20 mM sodium phosphate containing 0.005% polysorbate 80, pH 6.0. Triplicate extracts were analyzed for soluble aggregate content by anion exchange in series with size-exclusion chromatography (IEC-SEC), as described (20). The dimer and high molecular weight aggregate detection limits were 0.1%. Applying the extraction method to unencapsulated darbepoetin alfa powder showed no change in protein integrity by IEC-SEC compared with hydrated powder, with a recovery of 97.5 ± 1.5%. Encapsulation efficiency was expressed as recovered soluble monomer divided by total nominal darbepoetin alfa load.

The impact of low temperature solvent extraction on integrity was determined in duplicate by suspending darbepoetin alfa powder in cold ethanol (–80°C) and incubating at –40°C or –80°C. At various times, samples were equilibrated at –80°C for 45 min and centrifuged (4 min; 17,500 g) in a –80°C rotor. The sample temperature following centrifugation was less than –60°C. Sample tubes were transferred to dry ice and the supernatant removed. Pellets were dried by lyophilization and assayed by IEC-SEC as above.

In Vitro Release

Triplicate microsphere samples (20 mg) were suspended in 0.5 mL of sterile-filtered buffer (100 mM sodium phosphate, 10 mM sodium chloride, 0.005% polysorbate 80, pH 7.4) and incubated at 37°C, under static conditions. At 24 h and every 3–4 days thereafter, samples were centrifuged (4 min; 17,500 g) and the release medium replaced with fresh buffer. Sample manipulations were conducted in a laminar flow hood using sterile technique. Filtered (0.2-μm low-protein binding Aerodisk HT) samples were analyzed by IEC-SEC. The pH of the medium was confirmed to be 7.2 ± 0.2 through the study duration. Unreleased protein was recovered by extraction of the remaining microsphere mass as above. Extracts were reconstituted under denaturing conditions in 0.4 M Tris-HCl, 1% sodium dodecyl sulfate (SDS; pH 8.0), heated at 60°C for 10 min, and assayed for covalent aggregates by SEC using the same column as above, with a mobile phase consisting of 50mM sodium phosphate, 200 mM sodium chloride, and 0.05% SDS (pH 7.0). All protein quantities were expressed as a percentage of the initial protein present based on the theoretical load and the initial microsphere mass. Extracts were also analyzed by Western blot. After SDS-polyacrylamide gel electrophoresis (tris-glycine 14% polyacrylamide), samples were transferred by electroblotting to a polyvinylidene difluoride membrane, using the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA).

In Vivo Characterization

Pharmacokinetics and pharmacodynamics were evaluated in male NIHRNU-M nude rats (Taconic, Germantown, NY, USA), under a protocol approved by the Amgen Institutional Animal Care and Use Committee in compliance with the Principles of Laboratory Animal Care. Rats, 10–14 weeks old and weighing 323 ± 13 g, were housed in sterilized cages with sterile food and water and filtered air, and were handled in a laminar flow hood following aseptic technique. Animals (4 per group) were injected subcutaneously at the nape of the neck with the equivalent of 760 μg/kg darbepoetin alfa in microsphere form or as an aqueous bolus. Microspheres were suspended in a sterile vehicle containing 3% low viscosity CMC, 0.9% sodium chloride, and 1% polysorbate 80 in 25 mM sodium phosphate, pH 7.4. Microsphere samples (25 mg), which were not prepared under sterile conditions, were evaluated for bioburden by the USP pour plate method for total aerobic microbial count and for total combined mold and yeast detection (24). Blood samples were taken from the tail vein and complete blood count was measured using an Advia 120 (Bayer Corp, Tarrytown, NY, USA). Serum darbepoetin alfa levels were determined by ELISA using the Quantikine In Vitro Diagnostic rHuEPO immunoassay kit (R&D Systems, Minneapolis, MN, USA).

Data Analysis

Data were analyzed by analysis of variance using the JMP software package (SAS Institute Inc., Cary, NC, USA). Relative bioavailability was determined by normalizing area under the curve (AUC) of the darbepoetin alfa concentration profile for microsphere-treated animals to the AUC of a concurrent, darbepoetin alfa solution bolus control group; error represents a pooled standard deviation. AUC was calculated using the linear trapezoid rule.

RESULTS AND DISCUSSION

Physical Characterization

Two lots of darbepoetin alfa microspheres were fabricated by spray-freeze drying and three lots by spray drying. Encapsulation efficiencies were near 100% (Table I), consistent with other reports for both processes (10,14,21). Yields were higher with spray-freeze drying compared to spray drying (Table I). Darbepoetin alfa microspheres prepared by spray-freeze drying were found to be of irregular shape when examined by SEM (Fig. 2), as reported by others (21,25). Spray drying produced spherical particles, with no evidence of agglomeration or foreign particulates (26), or polymer fibers (27), as have been reported. For all microsphere prepa-

Table I. Properties of Darbepoetin Alfa Microspheres Prepared by Spray-Freeze Drying and by Spray Drying

Property	Process	
	SFD[a]	SD[b]
Encapsulation efficiency, %	95 ± 15[c]	103 ± 13
Yield, % (range)	73–82	34–57
Particle size, μm		
D[4,3]	29 ± 1	69 ± 5
Span	1.3 ± 0.1	1.1 ± 0.3
True density[d], g/mL	1.39 ± 0.02	1.30 ± 0.02
Bulk density[e], g/mL	0.62 ± 0.01	0.48 ± 0.01
Residual $MeCl_2$, ppm	<100	<500
Bioburden, CFU[f]	ND[g]	ND
Postencapsulation integrity (IEC-SEC), %[h]		
Monomer	98.9 ± 0.4	99.6 ± 0.1
Dimer	1.1 ± 0.4	0.4 ± 0.1
HMW[i]	ND	ND
In vitro release, %		
Cumulative at 24 h	23.5 ± 3.3	14.0 ± 0.7
Integrity of burst-release protein, %		
Monomer	99.2 ± 0.0	99.5 ± 0.4
Dimer	0.8 ± 0.0	0.5 ± 0.4
HMW	ND	ND

[a] SFD, spray-freeze dry; mean ± SD for n = 2 lots.
[b] SD, spray dry; mean ± SD for n = 3 lots.
[c] n = 4 lots.
[d] n = 1 lot for each process; mean ± SD for nine determinations.
[e] n = 1 lot for each process; mean ± SD for three determinations.
[f] CFU, colony-forming units.
[g] ND, not detected.
[h] Protein powder starting material was ≥99.6% monomer.
[i] HMW, high molecular weight.

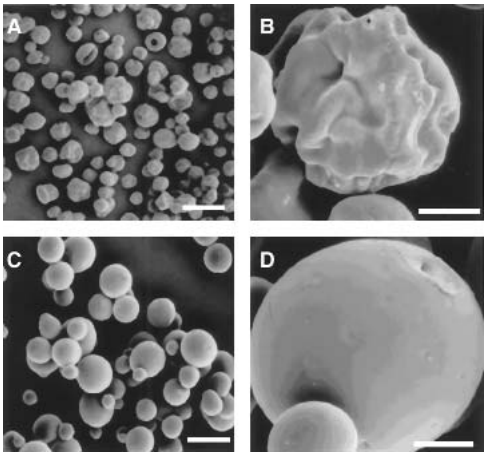

Fig. 2. Scanning electron microscopy of microspheres prepared by spray-freeze drying (A, B) and spray drying (C, D). Bar equals 50 μm (A, C) or 10 μm (B, D).

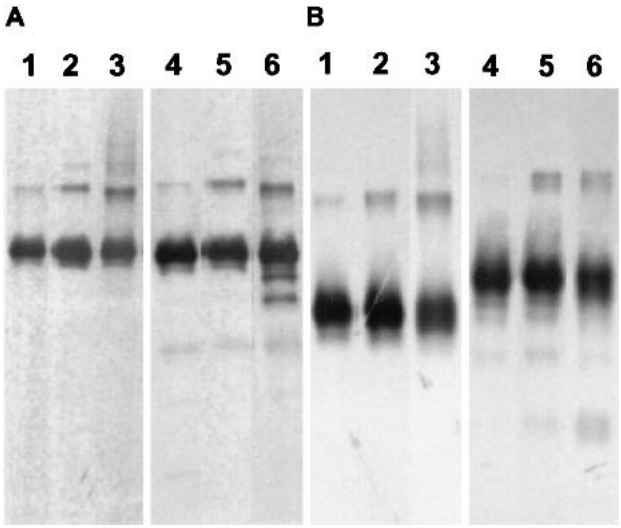

Fig. 3. Western blots of darbepoetin alfa control (lanes 1 and 4); darbepoetin alfa recovered from microspheres by extraction (lanes 2 and 5); and darbepoetin alfa remaining in microspheres after *in vitro* release for 4 weeks at 37°C, recovered by extraction (lanes 3 and 6). Microspheres were prepared by spray-freeze drying (A) or by spray drying (B). Samples were prepared under non-reducing (lanes 1–3) or reducing (lanes 4–6) conditions.

rations surface morphology was smooth, with few visible surface-accessible pores.

Microsphere size was about 2-fold larger in the case of spray drying compared to spray-freeze drying under the conditions tested (Table I). Particle size is a consequence of the size of the atomized droplets, their solids content, and the porosity of the dried product. The ultrasonic nozzles used for both processes produce droplets of comparable median diameter for atomized water, reported by the manufacturers to be 90-100 μm, but determined in this laboratory to be as low as 60 μm depending on the polymer, atomization power, and feed flow rate (data not shown). Internal morphology was difficult to assess by SEM, as both processes produced microspheres that were difficult to freeze fracture reproducibly. In select lots of spray-dried microspheres, hollow cores were observed (data not shown). Microsphere apparent true density was assessed and found for both processes to approximate that for bulk polymer (determined to be 1.36 ± 0.02 g/mL), indicating any pores were helium-accessible (Table I). Bulk density was determined, and found to be significantly lower for spray-dried microspheres compared to those made by spray-freeze drying (0.48 g/mL vs. 0.62 g/mL; Table I). A hollow-core structure, which can result from solvent evaporation and subsequent expansion of the nascent microspheres during spray drying (9), could be responsible.

Postencapsulation Darbepoetin Alfa Integrity

Postencapsulation darbepoetin alfa integrity was assessed for each of the processes by analyzing microsphere extracts by IEC-SEC and by Western blot. Soluble aggregate content was low in both cases, with <2% dimer and no high molecular weight aggregates detected by IEC-SEC in any of the lots tested. A small amount of covalent, non-reducible dimer was detected by Western blot in extracts of microspheres prepared by both processes (Fig. 3). In addition, Western blot revealed that spray-freeze dried microspheres contained a trace level of higher molecular weight aggregate, not detected by IEC-SEC (where the detection limit was 0.1%).

The excellent postencapsulation integrity after spray drying was consistent with previous results showing the subject darbepoetin alfa powder withstood methylene chloride exposure at room temperature for 30 min with no change in aggregate content (20). Although the spray drying inlet temperature of 55°C exceeds room temperature, evaporative cooling reduces the temperature of the atomized droplets. As drying commences the initial droplet temperature is approximately –9°C, as estimated from the wet-bulb temperature from the methylene chloride–nitrogen psychrometric chart (9). As solvent is removed, the product temperature reaches the outlet temperature of 28°C. Thus solvent exposure is transitory, at temperatures not far from ambient. In addition these temperatures are below the Tg values of many protein powders, which in general exhibit good stability toward organic solvent exposure (4). Although the lower cryogenic temperature range (–196°C to –80°C) in spray-freeze drying might increase the resistance of darbepoetin alfa powder to organic solvent exposure, drug particles are suspended in polymer solution at room temperature prior to atomization in both processes. When atomization is preceded by a particle size reduction step (as in the spray-freeze drying of human growth hormone; Ref. 3), room temperature solvent exposure time increases further.

Unexpectedly, postencapsulation integrity after spray-freeze drying was slightly lower than observed with spray drying for the specific processing conditions used here; soluble dimer content was approximately 0.7% higher (Table I). This slight but significant ($p < 0.0001$) difference prompted further evaluation of the two processes as applied to the present darbepoetin alfa formulation. (The formulation and protein dependence of this observation was not explored.) Both use methylene chloride at room temperature, but only spray-freeze drying requires an ethanol extraction step, ranging from hours to days (3). The impact of this step on integrity was evaluated by exposing darbepoetin alfa powder to ethanol under low temperature conditions, representative of the spray-freeze drying process (Fig. 1). Ethanol exposure re-

sulted in an increase in dimer content of 0.14 ± 0.02%, with no high molecular weight aggregates detected in any of the samples. Interestingly, dimer content was unaffected by the duration (up to 96 h) or temperature (–40°C or –80°C) of ethanol exposure. This result is possibly a consequence of water stripping by the organic solvent (4).

Differential residual solvent levels could possibly explain the slightly higher integrity with spray drying. Methylene chloride residual levels were low for both processes (Table I; Ref. 22). However, the ethanol content of spray-freeze dried microspheres was found to be approximately 1%. Residual ethanol could negatively impact storage stability, which was not evaluated.

Both of the processes examined here result in post-encapsulation integrity favorable compared to that reported by others for encapsulation of rHuEPO using emulsions. In the case of double emulsion (w/o/w) (28) aggregate levels were estimated from SDS-PAGE gels and found to vary with the formulation. No data on non-covalent aggregate content was reported. Covalent dimer and higher molecular weight rHuEPO aggregates were detected in microspheres made by spray drying a primary emulsion (14) but aggregate content was not quantitated. In contrast to these reports, rHuEPO integrity following encapsulation by spray-freeze drying (4) was comparable with that reported here for darbepoetin alfa.

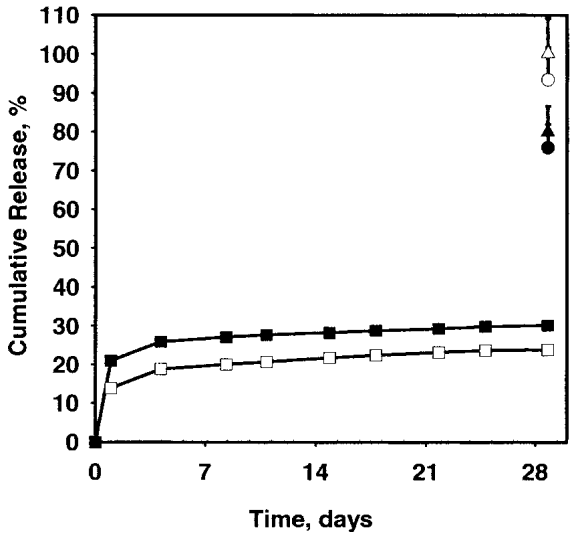

Fig. 4. *In vitro* release of darbepoetin alfa monomer (squares) from microspheres prepared by spray-freeze drying (closed symbols) and spray drying (open symbols). Variability was <2%. At the study conclusion, unreleased darbepoetin alfa was recovered by extraction and assayed by size-exclusion chromatography. Total cumulative monomer (circles) and cumulative protein (monomer plus aggregates; triangles) represent the sum of darbepoetin alfa recovered in the release medium and by extraction.

In Vitro Release

Darbepoetin alfa release from microspheres was characterized in a static test at 37°C. Burst-release results, defined as cumulative protein recovered at 24 h, appear in Table I. Nearly a 2-fold decrease in burst was observed for the spray dried microspheres, possibly due to their increased diameter (and hence decreased surface-to-mass ratio). Integrity of the burst-release protein was excellent for both processes, and compared favorably to the non-denaturing extraction results (Table I).

In vitro release kinetics was determined through 4 weeks for one lot from each process; results are shown in Fig. 4. For both processes, little protein was released following the initial burst. Total monomer recoveries were 30.2 ± 0.5% and 24.2 ± 0.2% for spray-freeze dried and spray dried lots, respectively. Dimer was below the detection limit for all *in vitro* release samples following day 4; no clips or high molecular weight aggregates were detected at any time point. The absence of significant protein release post-burst was consistent with minimal observed polymer mass loss (less than 10% of initial) over the course of the study.

To assess the integrity of unreleased protein at the conclusion of the study, darbepoetin alfa was extracted from the remaining microspheres and assayed under denaturing conditions for covalent aggregates by SEC. The accurate determination of non-covalent aggregates, which could be present in the extracts, was not possible due to the absence of a suitable extraction control. (This difficulty has been noted by others; Ref. 28.) Total monomer recoveries from *in vitro* release and postrelease extraction were 74 ± 6% for spray-freeze drying, compared to 91 ± 8% for spray drying, as calculated by SEC. Postrelease extracts, which were also assayed by Western blot, contained significant nonreducible covalent aggregates, with some clips apparent in spray-freeze dried material under reducing conditions (Fig. 3). Aggregate quantities were estimated from SEC results using the monomer standard curve, and expressed as percent of total initial protein. Dimer content for spray-freeze drying was about 3% lower than for spray drying (3.8 ± 0.7% vs. 6.5 ± 0.7%) whereas high molecular weight aggregate levels were comparable (1.9 ± 0.3% vs. 2.3 ± 0.5%). Total protein recoveries, as determined by summing SEC results for all darbepoetin alfa species detected, were 80 ± 7% (spray-freeze dry) compared with 100 ± 9% (spray dry). Overall, the two processes provide remarkably similar results under the conditions evaluated. The *in vitro* integrity results contrast with those of Morlock *et al.* (28) who characterized rHuEPO PLGA microspheres prepared by double emulsion. In an assay similar to that used here, covalent aggregates increased dramatically from 10% at day 1 to >40% at day 32.

In Vivo Characterization

The *in vivo* rate of darbepoetin alfa release from microspheres prepared by spray-freeze dry (one lot) and spray dry (three lots) processes was assessed in rodents by monitoring serum drug levels following a single injection (Fig. 5). Nude rats, which are athymic with limited antibody production, were used to avoid a confounding immune response to darbepoetin alfa, which is not an endogenous rat protein. (Others have used chemical immunosuppression to evaluate controlled release formulations of human protein therapeutics in rodents; Ref 21.) In select rat groups the absence of an anti-darbepoetin alfa immune response was confirmed by an insignificant antibody titer at day 39 (results not shown).

For all microsphere preparations tested, serum darbepoetin alfa levels remained elevated for over four weeks, approximately 3-fold longer than for unencapsulated darbepoetin alfa. Serum drug levels for microspheres prepared by both processes were essentially the same for the first two

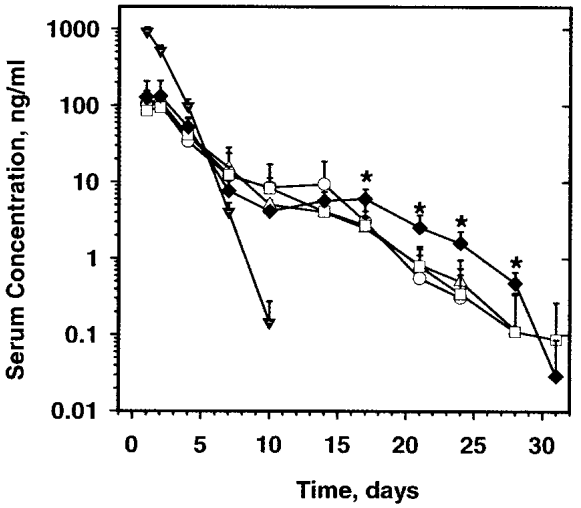

Fig. 5. Darbepoetin alfa serum concentration in nude rats after a single injection of darbepoetin alfa solution (▼) or of darbepoetin alfa microspheres prepared by spray-freeze drying (♦) or by spray drying (○, □, △). Asterisk depicts time points where microspheres prepared by the spray-freeze dry process differed significantly from each of the microsphere lots prepared by the spray dry process ($p < 0.05$ in each paired *t* test). At day 14, one spray dry lot (○) gave a slightly higher serum level than the other two ($p < 0.05$ for each paired *t* test).

weeks of the study. *In vivo* burst, as assessed by C_{max}, did not vary substantially among the microsphere preparations tested, ranging from 8–14% of the C_{max} observed for the bolus control. This contrasted with the *in vitro* burst results, where process dependence was observed (Table I). Consistency of microsphere *in vivo* performance among lots was quite good for spray dried preparations. Serum drug levels from the three lots tested were compared at each time point and found not to differ significantly from one another, with the exception of day 14 where minor variability was observed (Fig. 5). The *in vivo* rate of darbepoetin alfa release from microspheres, while not calculated, appears to be significantly higher than observed *in vitro* (Fig. 4). The difference could be explained by more rapid polymer hydrolysis, and subsequent erosion-controlled release of drug, *in vivo*. PLGA erosion rates are faster *in vivo* than *in vitro* (29). Furthermore, release rates are known to vary significantly with *in vitro* assay conditions (30).

Serum drug levels were significantly higher ($p < 0.05$) for microspheres prepared by spray-freeze drying during the third and fourth weeks of the study (Fig. 5). This difference resulted in a higher relative bioavailability of 28 ± 6%, compared with 21 ± 7% for all animals treated with spray dried microspheres ($p = 0.08$). The slightly higher dimer content observed in the *in vitro* release study of the latter may have contributed to this difference. The impact of formulation, which could be process dependent, was not evaluated. The most obvious difference among physical characteristics was particle size. Spray drying produced substantially larger microspheres, which might impact protein stability (for example, non-covalent aggregate content) in a manner not detected in the assays reported above. To test this hypothesis, microspheres prepared by spray drying were fractionated by manual sieving, resulting in batches with mean particle sizes of 34, 60, and 78 μm. The three batches were evaluated in rats as above, and serum darbepoetin alfa levels found not to vary. (Postencapsulation integrity was also found not to vary among the three fractionated samples.) For each process relative bioavailability was significantly below the total monomer recovered from *in vitro* release and post-release extraction (discussed above). Noncovalent aggregate formation during the course of release could be responsible.

To assess the impact of the process-dependence of darbepoetin alfa bioavailability, the duration of the pharmacodynamic effect, as indicated by blood hemoglobin concentration, was evaluated. Hemoglobin levels remained elevated for over seven weeks following a single injection of microspheres made by either spray-freeze drying or by spray drying, twice as long as observed with unencapsulated darbepoetin alfa (Fig. 6). The invariance of hemoglobin level with process demonstrates that the higher serum drug levels observed for spray-freeze dried darbepoetin alfa microspheres are of no consequence in this animal model. Additional work with other proteins will be required to determine if the slightly higher relative bioavailability observed with spray-freeze drying is a general finding. The impact of relative bioavailability on pharmacodynamic effect is likely protein dependent.

CONCLUSIONS

The present report demonstrates that for darbepoetin alfa under the conditions evaluated spray drying is an alternative to spray-freeze drying, which to date has been a preferred process for encapsulation of protein drugs. Postencapsulation integrity was slightly improved with spray drying. Although spray drying resulted in a slightly higher darbepoetin alfa covalent dimer content after *in vitro* release for 4 weeks, and in somewhat lower relative bioavailability, the duration of release *in vivo* matched that of spray-freeze drying, with an identical pharmacodynamic effect. Spray drying process robustness was demonstrated by excellent reproducibility of microsphere physical and chemical characteristics (Table I) as well as *in vivo* release kinetics (Fig. 5). Although spray drying has been considered inappropriate (3) for encapsulation of proteins, which are relatively thermolabile, the

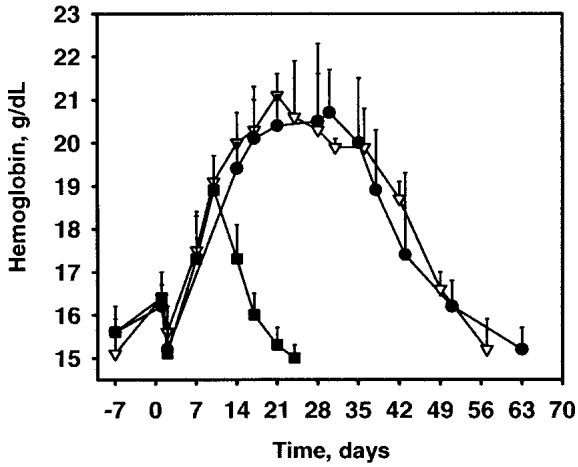

Fig. 6. Hemoglobin levels in nude rats after a single injection of darbepoetin alfa solution (■) or of darbepoetin alfa microspheres prepared by spray-freeze drying (●) or by spray drying (△).

temperature of methylene chloride exposure is similar to that required by spray-freeze drying, which additionally requires ethanol exposure. Nevertheless the generality of these conclusions will require testing with other proteins. The darbepoetin alfa particles encapsulated here were previously shown to withstand exposure to heat and to methylene chloride (20).

One important difference between the two processes as practiced here is scale. Spray drying was conducted using a commercially available pilot scale unit to attain residence times sufficiently long for fabricating microspheres large enough to fully encapsulate micron-sized drug particles. While the spray-freeze drying process is relatively uncomplicated at a bench-top scale, large-scale operation requires custom equipment. The pilot spray dryer used here could support clinical development, and possibly commercial production as well, for microsphere formulations of potent drugs. An area for further work is optimization of process yields, which at the 10 gm scale conditions evaluated here were low and variable compared to spray-freeze drying (Table I). Higher process yields would be required for successful commercial implementation.

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