The Stabilization and Encapsulation of Human Growth Hormone into Biodegradable Microspheres

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Received January 10, 1997; accepted March 4, 1997

Purpose. To produce and evaluate sustained-acting formulations of recombinant human growth hormone (rhGH) made by a novel microencapsulation process.

Methods. The protein was stabilized by forming an insoluble complex with zinc and encapsulated into microspheres of poly (D,L-lactide co-glycolide) (PLGA) which differed in polymer molecular weight (8–31kD), polymer end group, and zinc content. The encapsulation procedure was cryogenic, non-aqueous, and did not utilize surfactants or emulsification. The rhGH extracted from each of these microsphere formulations was analyzed by size-exclusion, ion-exchange and reversed-phase chromatography, SDS-polyacrylamide gel electrophoresis, peptide mapping, and cell proliferation of a cell line expressing the hGH receptor. In addition, the *in vivo* release profile was determined after subcutaneous administration of the microspheres to rats and juvenile rhesus monkeys.

Results. Protein and bioactivity analyses of the rhGH extracted from three different microsphere formulations showed that the encapsulated protein was unaltered relative to the protein before encapsulation. *In vivo,* microsphere administration to rats or monkeys induced elevated levels of serum rhGH for up to one month, more than 20-fold longer than was induced by the same amount of protein injected subcutaneously as a solution. The rate of protein release differed between the three microsphere formulations and was determined by the molecular weight and hydrophobicity of the PLGA. The serum rhGH profile, after three sequential monthly doses of the one formulation examined, was reproducible and showed no dose accumulation.

Conclusions. Using a novel process, rhGH can be stabilized and encapsulated in a solid state into PLGA microspheres and released with unaltered properties at different rates.

KEY WORDS: sustained-release; microencapsulation; microspheres; human growth hormone; poly (lactide *co*-glycolide); PLGA.

INTRODUCTION

Production of sustained-release delivery systems for therapeutic agents has a long history, and includes controlled-release oral formulations, transdermal patches, and implantable depot formulations (1). Several factors, however, have limited the development of sustained-release formulations of protein therapeutics. One is the need to stabilize the protein for long periods in an aqueous environment at physiological temperature. In contrast to lower molecular weight drugs, proteins often have large globular structures and exhibit secondary, tertiary, and, in some cases, quaternary structure that is necessary for their biological activity. In addition, proteins have many more labile bonds and chemically reactive groups on their side chains which can undergo oxidation (methionine, tryptophan, histidine, tyrosine), deamidation (arginine, glutamine) or disulfide reduction or interchange (cysteine).

One approach to development of sustained-acting formulations is to embed the drug into microspheres made of a biodegradable polymer (2–5). The homo- and co-polymers of lactic and glycolic acid (PLGA polymers) have numerous advantages for this application because they hydrolyze to give the acid monomers (2,3,6) and are chemically unreactive under the conditions used to prepare the microspheres. They are available in a range of molecular weights and monomer ratios thus providing a range of variables with which the release rate of the drug can be adjusted. They are non-immunogenic, well tolerated and non-toxic. In addition to their use in drug delivery (e.g. the depot formulation of the luteinizing hormone releasing hormone (LHRH) agonist, luprolide (7–9)), they are used to make biodegradable sutures, bandages, and bone plates (10–12).

It is important when producing microencapsulated formulations of therapeutic proteins, that the physical, chemical, and biological properties of the protein remain intact during encapsulation. It is particularly important that the protein not be altered in any way that will increase its immunogenicity. Such antibody responses can lead to safety concerns and, if neutralizing, can limit the efficacy of subsequent treatments. To avoid these problems we have stabilized rhGH by forming a zinc-protein complex and encapsulated the protein in the solid state into PLGA microspheres using a process that is conducted at cryogenic temperatures, employs no water and hence avoids oil-aqueous interfaces, and requires no surfactants. rhGH was chosen for these studies because it is currently administered by subcutaneous injection of protein solution either daily or three times a week to children with short stature caused by insufficient amounts of hGH (13,14). Recent clinical trials of continuous subcutaneous infusion of rhGH via a pump have shown that identical growth rates are achieved as when the protein is delivered by daily injection (15–18). These results demonstrate that administration of rhGH in a pulsatile fashion is not necessary for clinical efficacy.

We present herein both *in vitro* and *in vivo* analyses of different PLGA microsphere formulations of rhGH. Of these, three formulations, differing principally in the type of polymer used, were chosen for detailed analysis. The rhGH extracted from each of these microsphere formulations, as analyzed by chromatographic, electrophoretic, and bioactivity assays, was identical to the unformulated protein both after encapsulation and after release *in vitro.* Moreover, each of the formulations produced different serum profiles in rats thus showing it is possible to alter the release rate by rational selection of formulation variables.

MATERIALS AND METHODS

Microsphere Preparation

The fabrication procedure consisted of two steps originally described by Gombotz, *et al.* (19); in the first, the protein was

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0724-8741/97/0600-0730$12.50/0

formulated into a lyophilized powder and in the second, the lyophilized powder was encapsulated into the microspheres. To formulate rhGH (Nutropin, Genentech, Inc.), it was mixed with zinc acetate at a molar ratio of 6:1 zinc acetate:rhGH. The Zn:hGH dispersion was atomized through an ultrasonic nozzle into liquid nitrogen and the frozen droplets were lyophilized. The zinc-hGH powder and zinc carbonate were added to a solution of the polymer in dichloromethane and sonicated.

All microsphere formulations used D,L-PLGA (lactide:glycolide molar ratio of 50:50) and were obtained either from Birmingham Polymers (Birmingham, AL #115-56-1; 0.2 dL/g, 10kD, dodecanyl end group (denoted capped, Table I)) or from Boehringer Ingelheim (Ingelheim, Germany; RG502H, 0.2 dL/g, 8kD, and RG503H, 0.4 dL/g, 31kD, both of which had a carboxylic acid end group (denoted uncapped)). The polymer suspension was sprayed through a sonicating nozzle into a vessel containing frozen ethanol overlaid with liquid nitrogen. The vessel was then transferred to −80°C where the ethanol melted and the microspheres hardened as the dichloromethane was extracted by the ethanol. After 3 days, the microspheres were harvested by filtration and dried under vacuum and sieved through a 106 μm mesh screen.

Analyses of Microspheres and rhGH

Microspheres were placed on an aluminum stub and sputter coated with a layer of carbon or gold and imaged using a JEOL model 6400 scanning electron microscope. The mean particle diameter distribution of the microspheres was determined using a Coulter Multisizer. The hGH load of the microspheres was determined by nitrogen analysis.

The rhGH was recovered from the microspheres using two different methods. In the first, the protein was extracted by dissolving the microspheres in a mixture of methylene chloride and acetone and the protein precipitate was collected. In the second, the protein was recovered by incubating the microspheres in HEPES buffer. Chromatography was done according to published methods (20–24). Briefly, size-exclusion chromatography utilized a G2000SW XL TSK Gel Column with phosphate buffer as the mobile phase. Detection was by UV absorption at 214 nm. Reversed-phase chromatography utilized a polymeric reversed-phase column and acetonitrile gradient elution and was performed at 50°C. Ion-exchange chromatography was performed on a DEAE-5PW TSK Gel Column with a phosphate and acetonitrile gradient elution. Detection for the reverse phase and ion-exchange methods was by fluorescence with excitation at 286 nm and emission at 335 nm.

The bioactivity of hGH was determined using a cell proliferation assay using a cell line which expresses the receptor for hGH and proliferates in the presence of hGH (25). Cell proliferation was measured by conversion of Alamar Blue dye to a fluorescent product by intracellular reductases. rhGH containing samples were incubated with cells for 72 hr at 37°C, and fluorescence was quantitated which is proportional to the number of cells.

In Vivo Analyses

The microspheres were administered to Sprague-Dawley male rats or juvenile male rhesus monkeys (*Macaca mulatta*). In studies to evaluate the extended release of protein in rats, the animals were immunosuppressed by intraperitoneal treatment with cyclosporin and hydrocortisone (H. J. Lee, O. L. Johnson, *et al.*, unpublished). The microspheres (50 mg per rat or 50 mg/kg body weight per monkey) were suspended in an aqueous vehicle and injected subcutaneously. The rhGH solution used in these experiments was 13.2 mg/mL. Serum hGH concentration was determined using a radio immunoassay kit (ICN Biomedicals Inc., Costa Mesa CA). In the experiment where repeat doses of Formulation II (Table I) were administered to rats, three doses of microspheres (50 mg) were administered at 28-day intervals.

RESULTS

Microsphere Preparation and Formulation Variables

To stabilize the protein during the microsphere encapsulation process and within the microspheres after hydration, the protein was formulated with zinc acetate to produce a sparingly soluble Zn:rhGH complex. Zinc was chosen because histochemical analysis of the anterior pituitary shows that zinc ions are present in significant quantities in hGH secretory granules (26), and it is believed that hGH is stored in the pituitary as a zinc complex. In addition, rhGH complexed with zinc is more resistant than the monomer to denaturation with guanidine hydrochloride (27).

The microspheres were fabricated using a cryogenic, non-aqueous process (Figure 1) in which more than 95% of protein was incorporated into microspheres. The microspheres have a mean volume diameter of approximately 50 microns (Figure 2) and are shown in Figure 3 as a diagrammatic representation and by scanning electron microscopy. Eleven different microsphere formulations were made for initial evaluation (Table I). The variables that were investigated were polymer molecular weight, polymer end group, and the amount of zinc carbonate added as an excipient. The molar ratio of lactide to glycolide in all polymers was kept constant at 50:50 and the weight percent of Zn:rhGH in all microsphere formulations investigated was 6:1. The polymers had either a free carboxyl (uncapped) or an alkyl (C12) end-group (capped) and zinc carbonate was added to the polymer matrix to act as a depot for zinc ions to ensure that hGH remains complexed to zinc.

Results of Formulation Screening

The eleven microsphere formulations were assayed for protein aggregation by size-exclusion chromatography (SEC) and assessed *in vivo* by analysis of serum concentrations in rats after a single subcutaneous injection. *In vitro,* each of the formulations released protein that was essentially monomeric and matched the starting material (Table I) indicating that there was no effect of the formulation variables tested on protein aggregation. *In vivo,* however, the formulations performed differently. Of the three different polymers used, the formulations containing the 10kD capped end-group polymer had the highest average Cmax (1110 ng/ml) and the lowest average day 5 concentrations (4.8 ng/ml) whereas the formulations comprised of the 8kD uncapped end-group had the lowest average Cmax value (415 ng/ml) and induced the highest average Cday5 levels (14.2 ng/ml). This indicates that the lowest amount of protein was released initially, and the rhGH serum levels were most sustained, with microspheres comprised of the 8kD polymer. For formulations with the 10kD and the 31kD polymer, there

Table I. Characterization of rhGH PLGA Microspheres

Polymer	% Zinc Carbonate	% Monomer[a]	Cmax[b]	Cday5[c]
10 kD, capped	1	99.3	616 ± 384	4.5 ± 1.0
10 kD, capped	3	99.6	1050 ± 293	3.6 ± 0.8
10 kD, capped[d]	6	99.0	1670 ± 289	6.4 ± 1.3
8 kD, uncapped	0	99.3	323 ± 98.6	20.4 ± 14.2
8 kD, uncapped[e]	1	97.3	309 ± 67.1	9.0 ± 4.2
8 kD, uncapped	3	98.7	670 ± 244	18.8 ± 14.7
8 kD, uncapped	6	99.3	358 ± 58.9	8.5 ± 1.4
31 kD, uncapped	0	98.2	592 ± 318	4.5 ± 1.5
31 kD, uncapped[f]	1	98.8	433 ± 92	5.1 ± 0.3
31 kD, uncapped	3	99.4	644 ± 204	8.0 ± 2.6
31 kD, uncapped	6	99.8	1690 ± 340	6.6 ± 0.8

[a] % monomer in rhGH released *in vitro* at day 7 as determined by size exclusion chromatography; unencapsulated protein was 99.4% monomer by this assay.
[b] Average maximum serum rhGH concentration (ng/ml) after subcutaneous administration to rats. All values are mean ± standard deviation.
[c] Average serum rhGH concentration (ng/ml) at day 5.
[d] Chosen for further study (Formulation I).
[e] Chosen for further study (Formulation II).
[f] Chosen for further study (Formulation III).

was a trend toward a higher Cmax levels with a higher zinc carbonate content.

Because the most important factor in determining the serum rhGH profile was the polymer, three formulations, each using one of the three polymers, were chosen for further evaluation. These were: Formulation I: 6% zinc carbonate, 10 kD, capped polymer; Formulation II: 1% zinc carbonate, 8 kD, uncapped polymer; and Formulation III: 1% zinc carbonate, 31 kD, uncapped polymer.

In Vitro Analysis of rhGH in Selected Formulations

The physical and biological integrity of the protein recovered from the microspheres was analyzed by several complementary methods. Size exclusion (SEC), reversed-phase, and anion-exchange chromatography were used to detect aggregated, oxidized, and deamidated rhGH, respectively (13). These analyses (Table II) indicate that there were no significant differences between the protein before and after encapsulation. In addition, the specific bioactivity of the protein released from all three formulations was similar to that of the unencapsulated standard (Figure 4). In addition, SDS-reducing gel electrophoresis and HPLC analysis of a tryptic digest of Formulation II (data not shown) revealed that the extracted protein is comparable to unencapsulated protein. Taken together, these data show that the properties of the rhGH, either extracted or released from the microspheres after extended incubation at physiological temperatures, is unaltered.

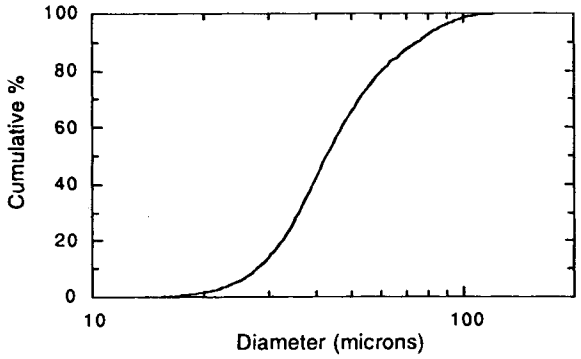

Fig. 2. Microsphere diameter distribution (by volume) of Formulation II. The median and mean diameters are 43.2 and 47.8 microns, respectively.

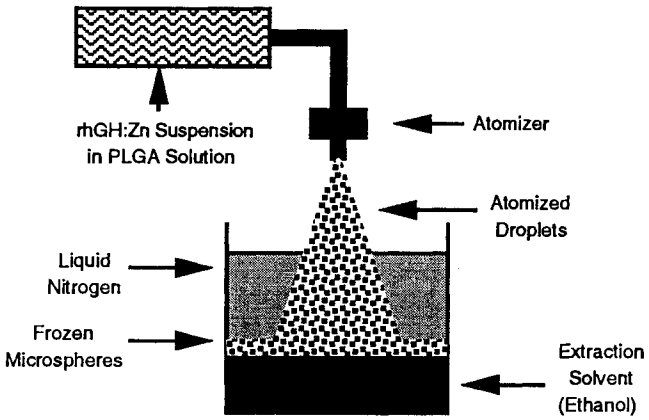

Fig. 1. Process for producing rhGH-containing PLGA microspheres. The Zn-rhGH complex is suspended in a solution of the PLGA polymer dissolved in dichloromethane and atomized through an ultrasonic nozzle and frozen. After the liquid nitrogen evaporates, the ethanol melts and extracts the dichloromethane from the microspheres which are then filtered and dried.

In Vivo Analysis of Selected Formulations

Each of the formulations was evaluated by measuring the hGH serum concentrations for several weeks after a single subcutaneous injection in juvenile rhesus monkeys and immunosuppressed rats (Figure 5A and B, respectively). In addition to the microsphere formulations, the monkeys (Figure 5A) or the rats (not shown) received the same amount of rhGH given as protein solution. All of the microsphere formulations showed an initial release phase of between 24–48 hr, during which the

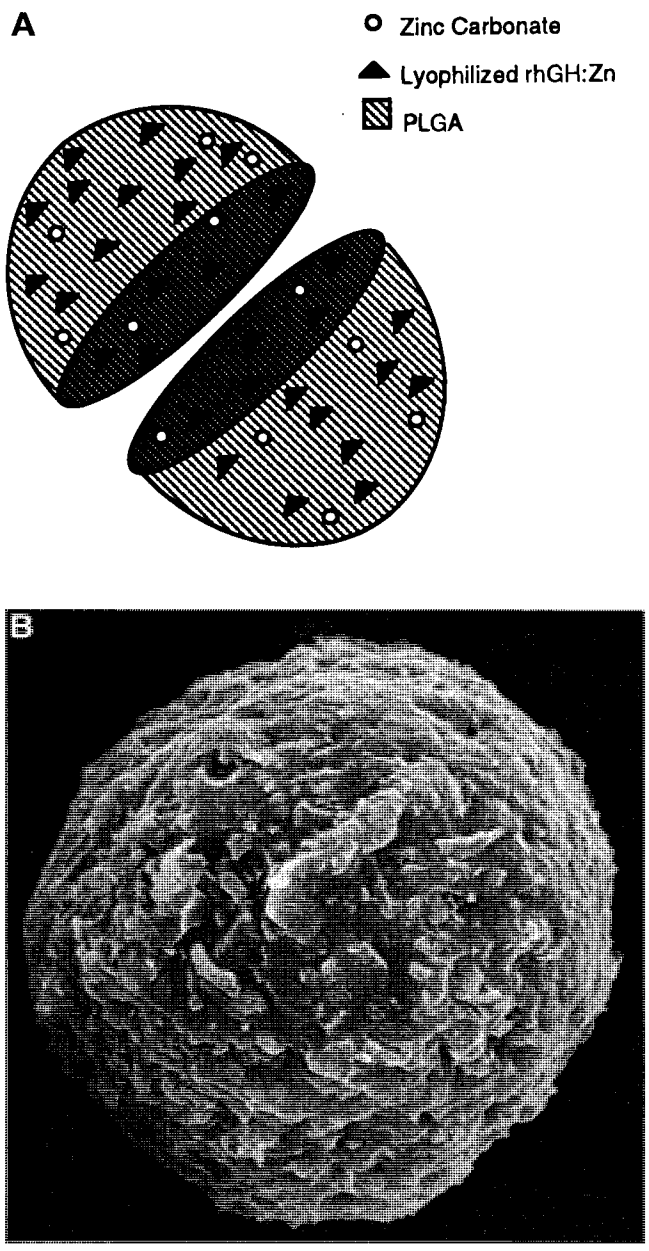

Fig. 3. A. Cartoon of internal structure of PLGA microsphere. The particles of rhGH:Zn complex and zinc carbonate, both of which are approximately 3 microns in diameter, are embedded into the solid PLGA matrix. The microspheres analyzed in our study were 15% w/w rhGH:Zn and between 0 and 6% w/w zinc carbonate. B. Scanning electron micrograph of a PLGA microsphere (Formulation II).

rhGH serum concentrations peaked at 8–12 hr after dosing (Figures 5A and B, inset). The Cmax of all formulations was more than six-fold lower than that induced by the protein in solution (280, 260 and 315 ng/mL for Formulations I-III, respectively, versus 2010 ng/mL for the rhGH solution in the monkeys).

After the initial release the concentrations dropped to more constant levels and all three formulations provided elevated serum hGH levels that were maintained above baseline (predose) concentrations for 3–4 weeks. During this period the serum levels induced by Formulations I and II were higher through about day 20 and day 10 in the monkey and rat, respectively. By contrast, the serum levels induced by Formulation III dropped more rapidly and were followed by an increase. To determine the serum levels induced by sequential administration of microspheres, three monthly doses of Formulation II were given to rats. The serum hGH profile after each monthly injection was similar for each of the three injections (Figure 6). This indicates that the performance of the formulation was reproducible from month-to-month and that there was no accumulation of hGH after sequential dosing.

Table II. Chromatographic Analysis of Encapsulated rhGH

	Size exclusion	Anion exchange	Reversed-phase
Unencapsulated	99.4%	99.0%	97.0%
Formulation I	98.7%	98.1%	96.8%
Formulation II	97.5%	97.7%	98.8%
Formulation III	98.5%	98.0%	99.0%

Note: Percent native protein as determined by each of the indicated chromatographic methods. The protein was recovered from each of the three microsphere formulations by dissolving the microspheres in a methylene chloride:acetone mixture, and recovering the insoluble protein. Size exclusion, anion exchange, and reversed-phase chromatography allowed identification dimers and higher order aggregates, deamidated, and oxidized rhGH, respectively (13).

DISCUSSION

The major roadblock to the development of sustained-release forms of proteins has been the ability to maintain protein integrity in a hydrated state at physiological temperature (28). Our results show that a zinc complex of rhGH can be encapsulated into PLGA microspheres and that the properties of the protein, both extracted from the microspheres and released *in vitro,* were unaltered. The most problematic altered form of rhGH is aggregate because, unlike oxidized or deamidated

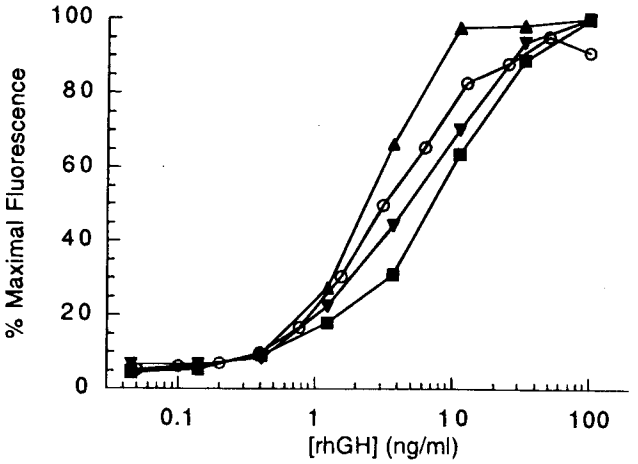

Fig. 4. Bioactivity of extracted rhGH measured by proliferation of a cell line expressing the hGH receptor. Microsphere Formulations I (■), II (▲) and III (▼) were incubated for 14 days in aqueous buffer and the concentration of protein released between days 10 and 14 was determined by SEC. Dilutions of these, as well as unencapsulated protein (○), were incubated with the cell line, Alamar Blue added, and fluorescence, which is an indication of cell number, was measured.

forms, aggregates are immunogenic and have reduced bioactivity (13). Thus, it is noteworthy that, even at the relatively high concentrations in the hydrated microspheres, the protein released from each of the three formulations is completely monomeric and fully bioactive. The reason for this may be that the protein remains in the solid state until it dissolves and is released.

The differences in the *in vivo* release profiles of the encapsulated hGH from the three microsphere formulations analyzed are consistent with what is believed to be the mechanism of drug release from PLGA microspheres (28–30). Release occurs by two principal mechanisms; i) diffusion of the drug from the surface of the microspheres during the initial release phase, and ii) release of the drug during the erosion of the polymer matrix during the sustained release phase. The initial release of the protein lasts up to 48 hr after injection and during this phase approximately 20% of the protein is released (31). Release of rhGH during this phase is via diffusion following dissolution of protein particles on the microsphere surface or of particles which, although not on the surface, have immediate access to the surface via a network of pores or channels within the polymer matrix.

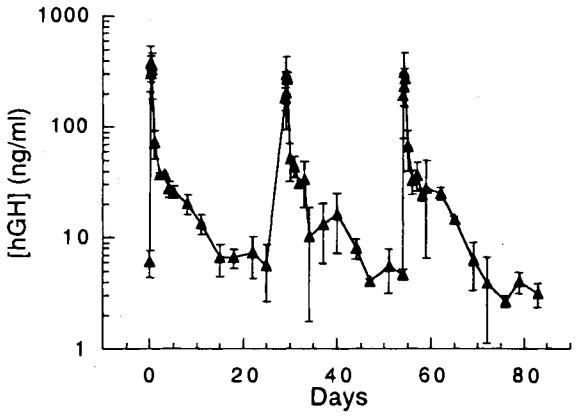

Fig. 6. Serum hGH levels in rats receiving three monthly formulations of Formulation II. Dosing and analysis was done as in Figure 5B.

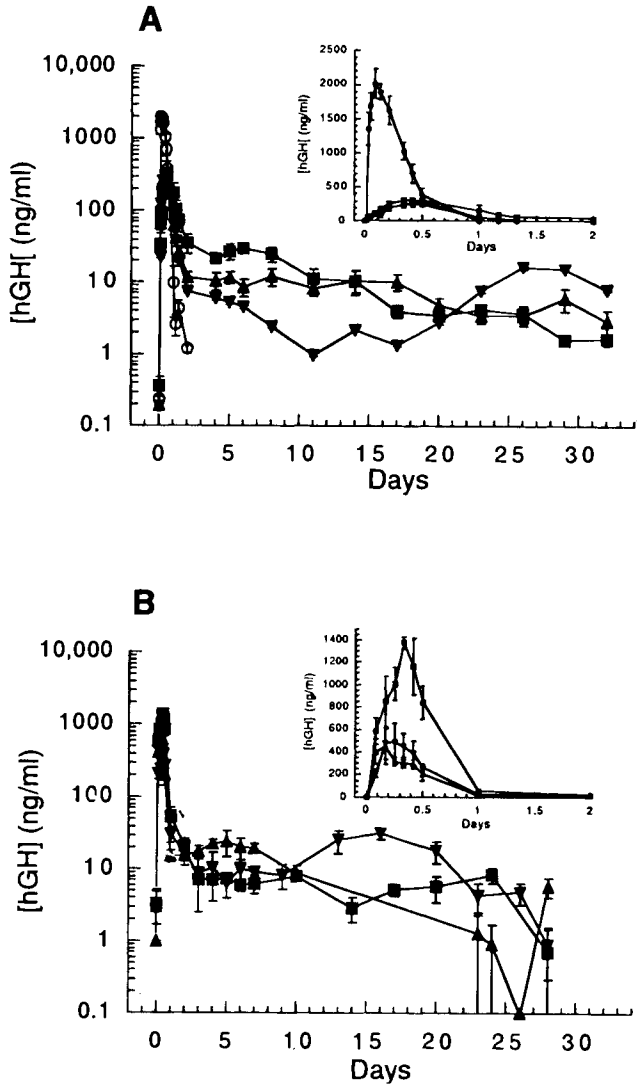

Fig. 5. Serum hGH levels of Formulations I (■), II (▲), and III (▼) after a single subcutaneous administration or either juvenile rhesus monkeys or rats. A. 50 mg/kg body weight of microspheres (7.5 mg rhGH/kg) were administered to juvenile rhesus monkeys. In addition, the monkeys received the same amount of rhGH in solution (○). B. 50 mg of microspheres (7.5 mg rhGH) were administered to immunosuppressed rats. hGH levels were determined in sera of both species by radioimmunoassay.

The release rate of protein during the second phase depends to a large extent on the properties of the PLGA polymer including molecular weight and the hydrophilicity/hydrophobicity ratio. During this phase protein release occurs principally by erosion of the polymer by hydrolysis to water soluble lactide-glycolide oligomers. The rate of degradation depends on the polymer molecular weight, with longer polymers requiring longer to degrade and release protein. The delay in the onset of the secondary release phase found with Formulation III may be explained by the slower degradation of the larger MW polymer (31 kD vs. 8 and 10 kD). With the higher molecular weight polymer, the drop in serum levels is due to the temporal separation of the diffusion-controlled phase and the erosion-dominant phase. One would also expect that increasing the hydrophobicity of the polymer by increasing the lactide content would prolong the lag phase (see, for example (32)). This has not yet been investigated with rhGH-containing microspheres.

The protein was not significantly altered in all of the formulations tested indicating that, at least among the three tested, the polymer type has no effect on protein integrity. This is not surprising given that the protein is present in the solid state throughout the microsphere fabrication process. In theory, any protein that can be stabilized as a solid can be encapsulated in a native form using this process. Compared to the three commonly used microencapsulation processes, phase separation, solvent evaporation, and spray drying, the process employed herein is superior in maintaining protein integrity for several reasons. First, after the protein is lyophilized there is no water present and hence no aqueous-organic solvent interfaces during the encapsulation process. Second, during the formation of the microspheres the protein remains at low temperatures until the microspheres are dried. And, finally, the protein incorporation efficiency is high because the protein-polymer suspension is sprayed in its entirety and the atomized droplets

eventually form the microspheres after dichloromethane extraction. The results presented herein provide encouragement that, in addition to rhGH, other therapeutic proteins can be stabilized and formulated into useful sustained-release systems with a variety of release characteristics.

ACKNOWLEDGMENTS

The authors thank Hye Jung Lee, Tracy Olson, Gary Riley, Julie Straub, Mark Tracy, Mark Wilson, Jim Wright, and Steve Zale for helpful discussions, Dennis Croll, Norman Kim, Suzie Lackey, Lynda Miller, Nan Dong, and Tony Pinho for technical assistance, and Robert Breyer, Michael Cronin, Robert Garnick, Alex Klibanov, Robert Langer, Rodney Pearlman, Richard Pops, and Bill Young for support and encouragement.

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