# The Stability of Recombinant Human Growth Hormone in Poly(lactic-co-glycolic acid) (PLGA) Microspheres

**Jeffrey L. Cleland,[1,6] Anne Mac,[1,2] Brooks Boyd,[1,3] Janet Yang,[1] Eileen T. Duenas,[1] Douglas Yeung,[1] Dennis Brooks,[1] Chung Hsu,[1] Herman Chu,[4] Venkat Mukku,[4] and Andrew J. S. Jones[5]**

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***Purpose.*** The development of a sustained release formulation for recombinant human growth hormone (rhGH) as well as other proteins requires that the protein be stable at physiological conditions during its *in vivo* lifetime. Poly(lactic-co-glycolic acid) (PLGA) microspheres may provide an excellent sustained release formulation for proteins, if protein stability can be maintained.

***Methods.*** rhGH was encapsulated in PLGA microspheres using a double emulsion process. Protein released from the microspheres was assessed by several chromatrographic assays, circular dichroism, and a cell-based bioassay. The rates of aggregation, oxidation, diketopiperazine formation, and deamidation were then determined for rhGH released from PLGA microspheres and rhGH in solution (control) during incubation in isotonic buffer, pH 7.4 and 37°C.

***Results.*** rhGH PLGA formulations were produced with a low initial burst (<20%) and a continuous release of rhGH for 30 days. rhGH was released initially from PLGA microspheres in its native form as measured by several assays. In isotonic buffer, pH 7.4 and 37°C, the rates of rhGH oxidation, diketopiperazine formation, and deamidation in the PLGA microspheres were equivalent to the rhGH in solution, but aggregation (dimer formation) occured at a slightly faster rate for protein released from the PLGA microspheres. This difference in aggregation rate was likely due to the high protein concentration used in the encapsulation process. The rhGH released was biologically active throughout the incubation at these conditions which are equivalent to physiological ionic strength and pH.

***Conclusions.*** rhGH was successfully encapsulated and released in its fully bioactive form from PLGA microspheres over 30 days. The chemical degradation rates of rhGH were not affected by the PLGA microspheres, indicating that the internal environment of the microspheres was similar to the bulk solution. After administration, the microspheres should become fully hydrated in the subcutaneous space and should experience similar isotonic conditions and pH. Therefore, if a protein formulation provides stability in isotonic buffer, pH 7.4 and 37°C, it should allow for a safe and efficacious sustained release dosage form in PLGA microspheres.

**KEY WORDS:** growth hormone; stability; poly(lactic-co-glycolic acid); microencapsulation; degradation; sustained release.

## INTRODUCTION

Previously, we described the successful encapsulation of recombinant human growth hormone (rhGH) in polylactic-coglycolic acid (PLGA) microspheres (1). This study demonstrated that rhGH in a stabilizing buffer formulation (trehalose or mannitol with phosphate buffer) may be encapsulated and released in its native bioactive form. This work characterized the initial release of rhGH from the PLGA microspheres and did not address the important issue of protein stability during release at physiological ionic strength, pH and temperature (e.g. isotonic buffer, pH 7.4, 37°C). Another recent study evaluated an insoluble rhGH formulation (zinc complex) in PLGA microspheres. This study demonstrated that rhGH formulated as a zinc complex was stable during microencapsulation and was released with a small increase in dimer formation over 28 days at 37°C (2). In previous stability studies, rhGH was observed to undergo deamidation, oxidation, and aggregation at physiological conditions (3). These degradation reactions did not affect the bioactivity of the protein, but were dependent upon the solution pH. Proteins in PLGA microspheres may be exposed to an acidic environment due to the water catalyzed hydrolysis of the polymer resulting in a different degradation rate than protein in a physiological buffer. However, a recent assessment of the pH inside PLGA microspheres revealed a constant pH of 6.3 for incubation at physiological conditions for over 40 days (4). This small decrease in pH from 7.4 to 6.3 may affect rhGH stability and solubility since rhGH (pI = 5.2) is less soluble at or near its isoelectric point (3).

To address the concerns regarding protein stability in PLGA microspheres, we chose to compare the rate of rhGH degradation for protein released from PLGA microspheres and protein in solution during incubation at 37°C in isotonic buffer, pH 7.4. If the previous hypothesis of an acidic internal environment is correct, the rate of rhGH degradation in the microspheres should be significantly different from the rate in solution. However, if buffer species and water readily penetrate the microsphere, the rates of degradation for microencapsulated rhGH and rhGH in solution should be equivalent. These studies provide valuable insight into the effects of PLGA on the stability of high purity recombinant proteins.

## MATERIALS & METHODS

### Materials

The rhGH was provided by Genentech Product Recovery Operations at 10 mg/mL protein in 10 mM ammonium bicarbonate, pH 7 and was then lyophilized to produce an excipient-free powder. 50:50 and 75:25 lactide/glycolide PLGA (RG502, RG752, RG756) were purchased from Boehringer Ingelheim. Polyvinyl alcohol (PVA; Airvol 205) was supplied by Air Products. Tween 20 (low peroxide), potassium phosphate, sodium hydroxide and sodium chloride were purchased from Mallinckrodt. Mannitol was obtained from Aldrich Chemicals. Methyl-

[1] Department of Pharmaceutical Research & Development, Genentech, Inc., South San Francisco, California.
[2] Current address: School of Pharmacy, University of California, San Francisco, California.
[3] Current address: Department of Chemical Engineering, University of California, Berkeley, California.
[4] Department of Quality Control, Genentech, Inc., South San Francisco, California.
[5] Department of Analytical Chemistry, Genentech, Inc., South San Francisco, California.
[6] To whom correspondence should be addressed. (e-mail: cleland@gene.com)

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ene chloride and acetonitrile were ACS grade from Baxter Healthcare Corporation. HEPES and sodium azide were obtained from Sigma Chemical Company.

### rhGH PLGA Microspheres

A double emulsion process was used to produce rhGH PLGA microspheres as described previously (1). Briefly, rhGH was formulated at 10 mg/mL protein in 5 mg/mL mannitol, 5 mM potassium phosphate, pH 8 and lyophilized. The lyophilized protein was reconstituted with 5 mM potassium phosphate to its maximum solubility (222 mg/mL rhGH, 111 mg/mL mannitol, 111 mM potassium phosphate, pH 8). For batch 1, 500 μL of the protein solution was injected into 4 mL of methylene chloride containing 2.3 g of PLGA (50:50 lactide/glycolide, 0.2 dL/g, 12 kDa; RG502, Boehringer Ingelheim) at room temperature while homogenizing at 7000 rpm. For batch 2, 600 μL of the protein solution was injected into the methylene chloride/PLGA solution. After homogenizing for 1 min, the water-oil emulsion was then mixed for 1 min with 300 mL of 6% w/v PVA in water saturated with methylene chloride (4.5 mL) at room temperature in a 1 L fermenter stirred at 800 rpm. Nascent microspheres were then hardened in 12 L of water for 1 hr. The final microspheres were filtered to remove large (>150 μm) and small (<20 μm) microspheres. The microspheres were washed with 15 L of water and then 15 L of 0.1% w/w Tween 20. Air drying of the microspheres was performed as described previously (5).

A polymer system comprising an equal mass of low (0.2 dL/g, 24 kDa, RG752) and high (0.6 dL/g, 100 kDa, RG756) inherent viscosity PLGA (75:25 lactide/glycolide, Boehringer Ingelheim) was used for batches 3 and 4. For batch 3, 750 μL of the protein solution was injected into 6 mL of methylene chloride containing 2.3 g of the polymers (1.15 g of each) and homogenized at 7000 rpm for 30 sec. The emulsion was then mixed with 300 mL of 6% PVA in water saturated with methylene chloride (4.5 mL) at 1200 rpm for 1 min. The resulting microspheres were then hardened and washed as described above. For batch 4, a larger scale of microsphere production was attempted. 2.25 mL of the rhGH solution was injected into 18 mL of methylene chloride containing 6.9 g of PLGA (3.45 g of each) and homogenized at 7000 rpm for 30 sec. The resulting solution was mixed at 1500 rpm with 900 mL of 6% PVA in water saturated with methylene chloride (13.5 mL) in a 1 L fermenter. The same hardening and washing process was again used. The final washed microspheres from batches 3 and 4 were resuspended with water and filled into vials. The vials were then lyophilized to produce a dry microsphere powder.

### rhGH Loading in Microspheres

The amount of rhGH encapsulated in the PLGA microspheres was determined by dissolving 20 mg of microspheres overnight in 1 mL of 1.0 N NaOH and measuring the absorbance at 284 nm ($E = 1.14\ (mg/mL)^{-1}cm^{-1}$).

### rhGH Release from Microspheres

The release of rhGH from PLGA microspheres was performed by using microcentrifuge eppendorf tubes (0.22 μm filters, PGC Scientific) as described previously (6). The retentate side of the filter unit was filled with 10–40 mg of rhGH PLGA microspheres or 10–40 mg of placebo PLGA microspheres. The microspheres were suspended with 300 μL of release buffer (10 mM HEPES, pH 7.4, 100 mM sodium chloride, 0.02% w/w Tween 20, and 0.02% w/w sodium azide). This buffer was sufficient to maintain the solution pH throughout the experiment due to the constant replacement of the buffer (every 1–2 days). The tubes were sealed with teflon-faced gray butyl stoppers (13 mm, West Company) and aluminum flip-top caps. Tubes were then placed on a rocker in a 37°C incubator. At time of sampling, the tubes were centrifuged at 13,000 rpm in a table top microcentrifuge for 1 min. The filtrate was removed and the microspheres were resuspended with 300 μL of fresh release buffer. The buffer addition was performed with a needle vent on the stopper (0.22 μm syringe filter) to prevent pressure build-up and maintain sterility. The amount of protein released was quantitated as described below. Protein concentration and sample volume were used to calculate the fraction of rhGH released at each timepoint. At the end of the release study, microspheres were centrifuged to remove excess water, resuspended in 1.0 N NaOH, and the remaining rhGH was quantitated by absorbance at 284 nm.

As a control for the rhGH PLGA microsphere release, rhGH was incubated at 1 mg/mL in the release buffer at 37°C. Individual samples were sacrificed at each timepoint and analyzed by the assays listed below.

### Analytical Assays for rhGH

#### *Quantitation of Released rhGH*

The amount of rhGH released from the PLGA microspheres was determined by using a bicinchoninic acid (BCA) assay (Pierce Chemical Company) in a microtiter plate format (6). Each set of samples were assayed with standards at rhGH concentrations between 5 and 1500 μg/mL. The values from the standards were fit to a four-parameter curve and the curve was used to calculate the rhGH concentration in the samples. All samples and standards were performed in duplicate on the same microtiter plate. Control studies with placebo microspheres in the release device indicated that the PLGA degradation products yielded a background concentration of 20 μg/mL initially (1 hr) and a consistent background of approximately 2.5 μg/mL in the BCA assay over extended incubation at 37°C. Approximately one half of this background effect was contributed by the release device (filter system). The sample concentrations were therefore corrected for this background effect. Protein release from the microspheres usually consisted of at least 1 mg/mL rhGH in the initial burst (1 hr) samples and 20 μg/mL rhGH in the later timepoints.

#### *Sixe Exclusion Chromatography (SEC)*

The amount of soluble rhGH aggregates in each sample were quantitated by SEC HPLC. A Zorbax GF250 (DuPont Chemical Company) or a TSK 2000 SWXL (Toso-Haas) column was operated at 1.0 mL/min using a mobile phase of 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 on an HPLC system (1090 L, Hewlett Packard). Protein peak detection was performed at 214 and 280 nm. The relative peak area of monomeric rhGH and soluble aggregates was measured and the total peak area was compared to control rhGH samples

of known concentration to quantitate release of monomeric rhGH (1).

*Reversed Phase HPLC (RP HPLC) at Neutral pH*

The oxidation of rhGH and generation of the des-PhePro rhGH was assessed by RP HPLC as described previously (7,8). A PLRP-S column (4000 Å, Polymer Laboratories) was operated at 40°C on a HPLC with a binary gradient consisting of 50 mM sodium phosphate, pH 7.5 (buffer A) and 100% acetonitrile (buffer B). The minimum protein loading was 2 μg and the flow rate was 1.5 mL/min over 21 minutes. The gradient consisted of 25% to 35% acetonitrile in 3 min, followed by a gradient to 47% acetonitrile for 9 min. Any remaining protein was eluted with a step to 70% acetonitrile for 3 min and then the column was equilibrated to 25% acetonitrile for 6 min. The peak elution was monitored at 214 and 280 nm.

*Anion Exchange Chromatography*

The amount of deamidated rhGH was quantitated by anion exchange chromatography (9). A TSK DEAE-5PW column (Toso-Haas) was used with a binary solvent system consisting of 180 mM potassium phosphate, 10% acetonitrile, pH 7.4 (buffer A) and 10% acetonitrile, pH 7.4 (buffer B). The column was operated at 0.5 mL/min at 45°C. A linear gradient from 50 to 100 mM potassium phosphate was performed over 25 min. Native and deamidated rhGH were quantitated by measuring the fractional peak areas as detected by absorbance at 214 and 280 nm.

*High Performance Receptor Binding Chromatography (HPRBC)*

To assess the ability of rhGH to bind to its receptor, a chromatographic assay was used. This assay required the incubation of rhGH and its soluble receptor, growth hormone binding protein (GHBP) and analysis of complex formation. Studies indicated that by maintaining equal ratio of GHBP and rhGH at low total protein concentration, the binding of rhGH could be quantitated by measuring the formation of the rhGH-GHBP complex (2 GHBP: 1 rhGH) (10). The amount of complex, free rhGH and free GHBP were quantitated by SEC HPLC using a TSK 3000 SWXL (Toso-Haas) column with a mobile phase of 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 running at 1.0 mL/min.

A further confirmation of rhGH biological activity was performed using a cell-based assay. This assay requires rhGH binding to two GH receptors (receptor dimerization) on the cell surface to cause cellular proliferation as described previously (10). Samples were assayed as described in the previous studies (1,10). Further confirmation of the native structure of rhGH was performed by ELISA and circular dichroism (CD) assays as detailed in a previous study (1).

## RESULTS AND DISCUSSION

### rhGH PLGA Microsphere Properties

The preparation of rhGH PLGA microspheres was performed by using a typical water-in-oil-in-water emulsion process. Microspheres were first produced with a low inherent viscosity 50:50 lactide/glycolide PLGA (0.2 dL/g; 12 kDa). For batch 1, this process yielded a protein loading of 2.0% w/w rhGH. Based upon the theoretical loading (e.g. amount of protein added in first emulsion/total mass of polymer and protein), a 50% encapsulation efficiency (actual/theoretical) was achieved for this batch. In an attempt to achieve a higher loading, slightly more protein was added to the first emulsion in batch 2. However, this batch had the same protein loading (2.0% w/w) and therefore had a lower encapsulation efficiency (40%). As shown in Figure 1, both of these preparations provided a low initial burst (1 hr) and a continuous release of rhGH over time at 37°C in the release buffer (isotonic, pH 7.4). A slightly faster release and higher initial burst were observed in batch 2 (Figure 1), and this release may have resulted from the larger aqueous volume used in the first emulsion. Similar effects of first emulsion aqueous volume on release rate have been observed, where increasing the aqueous volume increased the release rate (5,11).

To achieve higher protein loading and encapsulation efficiency, microspheres were prepared with a 50:50 mass ratio of low (0.2 dL/g; 24 kDa) and high (0.6 dL/g; 100 kDa) inherent viscosity 75:25 lactide/glycolide polymers. For batch 3, a protein loading of 3.8% w/w rhGH was obtained with an encapsulation efficiency of 66%. These results indicated that an increase in encapsulation efficiency may be achieved by mixing low and high inherent viscosity PLGA as previously observed (5). A larger scale preparation, batch 4, was made with the same polymers and theoretical protein loading. This batch had a protein loading of 3.7% w/w rhGH and an encapsulation efficiency of 63%, revealing that this process may be readily scalable without significant losses in protein loading or efficiency.

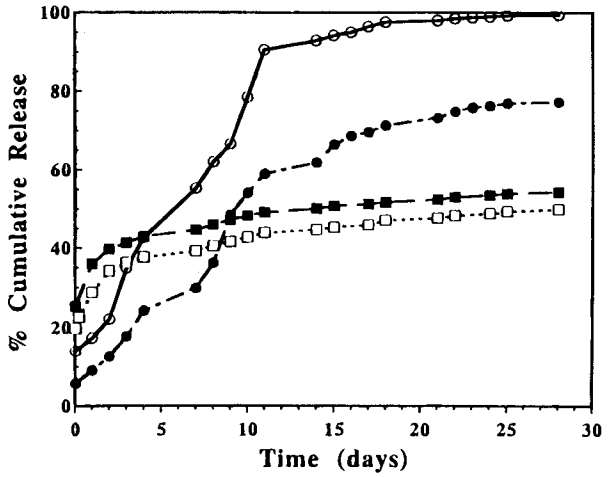


**Fig. 1.** The *in vitro* release of rhGH from PLGA microspheres was performed by incubation at physiological conditions (pH 7.4, 37°C). Each data point was generated by removal of the release buffer and quantitation of the protein concentration by the BCA method (see Material & Methods). Microspheres were produced with a low inherent viscosity (0.2 dL/g) 50:50 lactide/glycolide PLGA (batch 1: closed circles, batch 2: open circles) or with a 50:50 mass ratio of low (0.2 dL/g) and high (0.6 dL/g) inherent viscosity 75:25 lactide/glycolide PLGA (batch 3: closed squares; batch 4: open squares). The samples were sacrificed at 28 days and the remaining protein was quantitated by dissolution in 1 N sodium hydroxide. Greater than 90% mass balance was achieved for each batch. The Day 0 release shown in the graph represents the protein released after 1 hr of incubation.

The release profiles from both of these preparations displayed an initial release phase followed by a period of little or no release. The reduced release rate may be the result of a slower erosion phase for these polymers. As discussed previously, a 75:25 PLGA degrades more slowly than a 50:50 PLGA and the time to bulk erosion increases with increasing molecular weight (12). The initial and subsequent release was similar for both the small and large scale processes. The high initial burst (>20%) for both of these batches was likely caused by the lyophilization process. Previous work revealed that lyophilization of microspheres produced with this process may result in cracking of the microspheres leading to a larger initial burst (10).

### Quality of Initial rhGH Released

The microencapsulation process may often result in significant denaturation of the protein and the initial protein released may be an accurate representation of the encapsulated protein (1). The initial protein released (1 hr, 37°C) from the PLGA microspheres was characterized by SEC HPLC, HPRBC, ELISA, and CD assays. As shown in Table 1, the protein released in the initial burst contained a similar amount of monomeric rhGH as the untreated control. However, release of monomeric rhGH does not guarantee an intact native structure since rhGH may be denatured upon exposure to methylene chloride (1). The surface structure of rhGH was assessed by antibodies that bind to the epitopes on the rhGH. ELISA results indicated that the antibody binding was unchanged after encapsulation (Table 1). To assure that the receptor binding epitope was maintained intact, the HPBRC assay was performed on the initial protein released from the microspheres. These results confirmed that the receptor binding epitopes on rhGH were maintained after encapsulation.

Further physical characterization of the released rhGH was done to assess the protein's secondary and tertiary structure. The secondary structure of rhGH released initially was unaltered as measured by far ultraviolet CD (Figure 2). In addition, near ultraviolet CD spectra revealed that there was no significant change in the tertiary structure of rhGH released in the initial burst. Based upon these results along with the results from other assays, rhGH released initially from the PLGA microspheres had a native protein structure. In addition, the lack of batch-to-batch variability in these results suggests that the encapsulation process and polymer type (lactide/glycolide ratio and inherent viscosity) do not affect the protein stability.

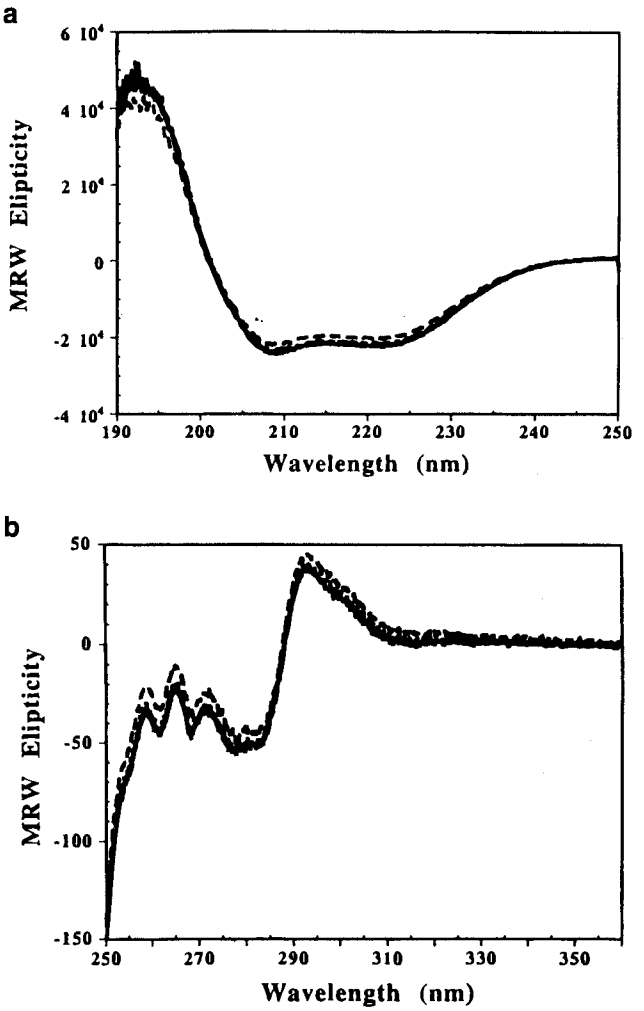


**Fig. 2.** Circular dichroism (CD) analysis of rhGH initially (1 hr incubation) released from PLGA microsphere batches 1 through 3 (see Figure 1). A control sample (—) in the release buffer was also analyzed by both far (a) and near (b) ultraviolet CD. rhGH released initially from batches 1 (——), 2 (---), and 3 (—·—) were not significantly different from the control sample in this assay.

**Table 1.** Quality of rhGH Released After 1 Hour Incubation at 37°C

| Sample | % Monomer[a] | ELISA[b] | HPRBC[c] |
|---|---|---|---|
| Batch 1 | 97 | 123 | 99 |
| Batch 2 | 92 | 110 | 97 |
| Batch 3 | 98 | 120 | 97 |
| rhGH | 98 | 112 | 97 |

[a] % monomer was defined as the fraction of the total peak area by SEC-HPLC that corresponds to the monomeric form of rhGH. The remaining fraction was rhGH dimer.
[b] ELISA results were calculated as a percentage of the reference material (100%) run in the same assay.
[c] High performance receptor binding chromatography (HPBRC) was performed on each sample and the results are presented as a percentage of the reference material (100%).

### Stability of rhGH Released from PLGA Microspheres

For a successful sustained release formulation, the protein must be released in its native form both initially and during its lifetime *in vivo*. The microspheres will be exposed to a complex millieu of ions and macromolecules *in vivo* (13). While the macromolecules (e.g. proteases) can not have an impact on the encapsulated protein since they can not diffuse into the microspheres, ions (e.g., buffer salts) can diffuse into the fully hydrated microspheres and affect the protein stability. To simulate physiological ionic strength and pH, we chose 10 mM HEPES, pH 7.4, 100 mM sodium chloride, 0.02% w/w Tween 20, and 0.02% w/w sodium azide as the release buffer. The surfactant was used to prevent protein adsorption to the release device and the sodium azide was used to maintain sterility, even though all studies were done under sterile conditions. The

rhGH released from the PLGA microspheres incubated at 37°C in this buffer was analyzed by stability indicating HPLC assays to determine the rates of aggregation, oxidation, and deamidation over 28 days of release. In addition, the biological activity of rhGH released from the microspheres was measured. To assess the effect of the PLGA microsphere environment on protein stability, 1 mg/mL rhGH in the release buffer as a control was incubated at 37°C and analyzed over the same time period in the same assays.

The initial protein released from the PLGA microspheres (1 hr) was equivalent to the control protein in the SEC, RP, and anion exchange HPLC assays (data not shown). The protein released after the initial burst was quantitated in each of the assays and the rate of protein degradation was determined by a first order exponential fit to the decrease in the non-degraded protein peak fraction. As shown in Table 2, the rate of rhGH aggregation was faster for the protein released from the PLGA microspheres as compared to the control solution. After 18 days at 37°C, the control sample contained 2% dimer and the microsphere sample was 18% dimer. Larger aggregates were not observed over the course of this study. A more rapid rate of aggregation may be caused by the high concentration of the protein encapsulated in the microspheres (~200 mg/mL) compared to the low concentration of the control sample (1 mg/mL). Measurement of the protein remaining in the microspheres at the end of the study demonstrated that greater than 90% of the protein was recovered with the remainder likely due to cumulative assay errors (BCA and loading assays). Therefore, these results accurately represent the protein released from the PLGA microspheres.

Unlike the aggregation rates, the rate of oxidation was comparable for protein released from the microspheres and protein in the control sample (Table 2). For all samples, rhGH was observed to undergo methionine oxidation and degrade via a diketopiperazine reaction (des-PhePro). The oxidized and degraded rhGH forms have been extensively characterized (3,7,8). The rate of rhGH oxidation has been previously observed to be insensitive to pH at 40°C (B. O'Connor and J. Q. Oeswein, unpublished results). The deamidation of rhGH occurred more rapidly than the oxidation. The deamidation rate for batch 2 was unusually high, while the rates for the other batches were less than the control sample. Previous studies have shown that rhGH deamidation is very sensitive to pH and the rate of deamidation at 40°C was two fold slower at pH 6.0 than pH 6.5, but only approximately 30% faster for each increment of 0.5 pH units above 6.5 (3,9). Thus, if the pH inside the microspheres is lower than the bulk solution, then deamidation would occur more slowly and the succinimide intermediate would be stabilized. The data shown in Table 2 reveal a general trend toward lower deamidation rates (except batch 2) than the bulk solution, but are not statistically significant when compared to the control sample. Also, rhGH solubility decreases dramatically as a function of decreasing pH from 8.0 to 5.0 (3), but the rate and extent of aggregation do not indicate a large decrease in solubility. Taken together, these results suggest that the pH within the hydrated PLGA microspheres is not significantly different from the pH of the bulk solution. Therefore, the continuous influx of fresh buffer into the microspheres may prevent a decrease in pH that could result from the liberation of acid during PLGA hydrolysis. A recent study that measured pH within PLGA microspheres further confirmed that the pH does not decrease dramatically inside the hydrated microspheres during PLGA hydrolysis (4).

**Table 2.** Degradation of rhGH in Solution and Released from PLGA Microspheres Incubated at 37°C

| Sample[a] | Aggregation[b] | Oxidation[c] | Deamidation[d] |
|---|---|---|---|
| Batch 1 | 0.027 ± 0.007 | N.D.[e] | 0.027 ± 0.008 |
| Batch 2 | 0.009 ± 0.0006 | 0.006 ± 0.002 | 0.059 ± 0.006 |
| Batch 3 | N.D. | 0.010 ± 0.004 | 0.033 ± 0.009 |
| Batch 4 | 0.013 ± 0.003 | 0.006 ± 0.002 | 0.029 ± 0.007 |
| rhGH | 0.002 ± 0.0002 | 0.009 ± 0.002 | 0.040 ± 0.007 |

[a] Samples were incubated at 37°C in physiological buffer and replenished frequently. Released protein was quantitated by BCA and analyzed by each method. Rate constants ($d^{-1}$) for each degradation reaction were calculated from a single exponential decay of the intact native rhGH fraction remaining. The control (microspheres and untreated rhGH) were also incubated under the same conditions.
[b] Aggregation was measured by SEC-HPLC. Aggregates larger than dimers were not detected in samples.
[c] Oxidation was detected by neutral pH reverse phase HPLC.
[d] Deamidation was assessed by ion exchange HPLC.
[e] N.D. = not determined.

A one month continuous release formulation of rhGH PLGA microspheres would result in release of degraded protein *in vivo*. From the results in Table 2, rates of aggregation, oxidation, and deamidation may be estimated to be 0.01, 0.007 and 0.037 $d^{-1}$, respectively. After 30 days at 37°C and physiological pH, the release rhGH would then be 74% monomer (remainder dimer), 82% non-oxidized, and 35% non-deamidated. These levels of degradation would not be acceptable for the storage of a protein pharmaceutical. However, this degradation would take place after administration, and it is likely that the hGH stored in secretory granules in the pituitary is exposed to similar conditions for extended period (3).

The release of degraded rhGH is acceptable only if it does not affect the protein's biological activity (efficacy) and immunogenicity (safety).s The biological activity of rhGH released from the PLGA microspheres over time was measured by an *in vitro* cell-based assay. The protein relased over 21 days *in vitro* was fully bioactive within the error of the assay (± 20%; Figure 3). Only one spurious sample (day 15, batch 1) had less than 90% bioactivity. The protein degradation also did not affect the immunogenicity as described previously (14). Therefore, although rhGH undergoes degradation while at physiological pH, ionic strength, and temperature, this degradation does not appear to affect its activity or immunogenicity.

## CONCLUSIONS

For the successful clinical application of a sustained release dosage form, the drug must be released from the device (e.g. microspheres) in its bioactive form during the *in vivo* lifetime of the device. A biodegradable system such as PLGA microspheres provides the opportunity for continuous release of protein drugs over extended periods (days or months). However, the degradation of the PLGA microspheres results in the generation of lactic and glycolic acid that may affect the protein stability. Furthermore, many proteins are intrinsically unstable for long periods (days) at physiological conditions.

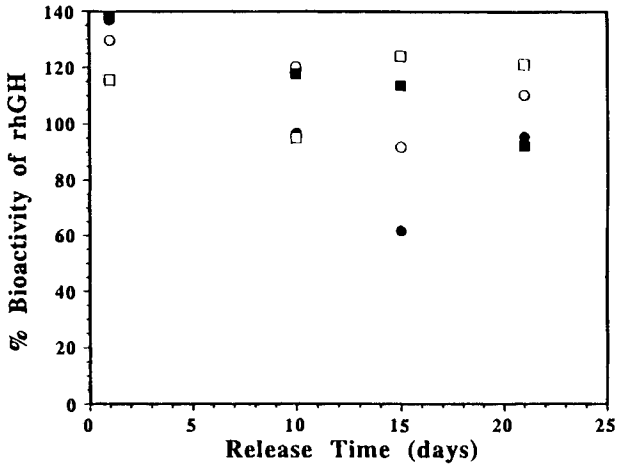


**Fig. 3.** rhGH released from the PLGA microspheres incubated at physiological conditions was measured by the *in vitro* cell-based bioassay. The protein concentration measured by the bioassay with the appropriate rhGH standards was compared to the protein concentration measured by the BCA assay (% bioactivity = [rhGH] by bioassay / [rhGH] by BCA * 100%). rhGH released from batch 1 (closed circles), 2 (open circles), 3 (closed squares), and 4 (open squares) was assayed after a given incubation time.

In these studies, we have assessed the stability of rhGH during release from PLGA microspheres at physiological pH, ionic strength, and temperature. The protein was successfully encapsulated and continuously released in its native form as measured by spectral and chromatographic assays. These studies also indicated that the different process conditions and polymers used for each batch did not affect the protein quality. The rhGH in the PLGA microspheres aggregated to form dimers at a slightly faster rate than the control rhGH (not encapsulated) in the release buffer. This rate of aggregation was also slightly greater than that observed with a zinc-rhGH complex formulation in PLGA microspheres (2). The use of a concentrated (222 mg/mL) rhGH solution in the first emulsion probably led to the greater rate of aggregation. In contrast, oxidation and deamidation rates were not significantly different between the PLGA microsphere and control samples, indicating that these reactions were not affected by the PLGA. Previous work has shown that oxidation (3,7), diketopiperazine formation (3,8), and deamidation (3,9) in rhGH are sensitive to solution pH. If rhGH was in an acidic environment, its deamidation and oxidation rates should have been significantly less than those observed at pH 7.4. Therefore, these results indicated that the aqueous environment inside the PLGA microspheres is similar to the bulk solution and the continuous replacement of the bulk solution provides sufficient buffering capacity for the acid generated by PLGA hydrolysis. Overall, it is reasonable to hypothesize that the *in vivo* conditions should provide a constant buffer reservior and thereby, maintain the physiological pH within the PLGA microspheres.

The rates of rhGH degradation at physiological conditions resulted in significantly degraded rhGH (74% monomer (remainder dimer), 82% non-oxidized, and 35% non-deamidated) after 30 days. The biological activity (cell-based bioassay) of rhGH was however maintained throughout the duration of its release from the microspheres (Figure 3) and the released rhGH was not immunogenic in primates (14,15). Based on these results, this rhGH PLGA formulation should provide a safe and efficacious method for sustained delivery over 30 days.

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