Formation of Acylated Growth Hormone-Releasing Peptide-6 by Poly(lactide-co-glycolide) and Its Biological Activity

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ABSTRACT

The purpose of this study was to investigate the formation of acylated impurity resulting from a chemical reaction between the growth hormone-releasing peptide-6 (GHRP-6) and poly(lactide-co-glycolide) (PLGA) and the effect of peptide acylation on the in vivo biological activity of GHRP-6. The peptide acylation pattern of GHRP-6 by hydrophilic PLGA polymers with different molecular weights was characterized by reversed-phase high-performance liquid chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Higher levels of acylated GHRP-6 were produced with the higher molecular weight PLGA, which might be due to the slower degradation rate of the polymer. The evaluation of the biological activity in rats showed that the acylated GHRP-6 had a much lower activity than the intact GHRP-6. This finding suggests that the acylation reaction would decrease the effectiveness of the GHRP-6 formulation such as PLGA microspheres. Therefore, a strategy for stabilizing the GHRP-6 will be necessary for the development of a successful formulation of PLGA microspheres.

KEYWORDS: Poly(lactide-co-glycolide), microspheres, peptide acylation, stability, growth hormone releasing peptide-6.

INTRODUCTION

Poly(lactide-co-glycolide) (PLGA) has been extensively used for drug delivery and tissue engineering on account of its highly biocompatible and biodegradable characteristics.[1,2] Over the last 2 decades, various peptides and proteins have been successfully incorporated into PLGA microspheres using different formulation methods.[3-5] In addition, sustained depot formulations of the luteinizing hormone-releasing hormone and somatostatin analogs have been commercialized, and several additional products are under clinical investigation. However, the instability of peptides and proteins in the PLGA microspheres during the manufacture and storage, or after administration are still major challenges to successful product development.[6,7]

Peptide acylation is one of the peptide destabilization mechanisms in PLGA microspheres.[7] Lucke et al reported evidence of peptide acylation in the PLGA microspheres containing salmon calcitonin and atrial natriuretic peptide by using a liquid chromatography-mass spectrometry (LC-MS) technique.[8] By monitoring the stability of the 3 peptides in PLGA microspheres by capillary electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), Na et al determined that the primary amines (N-terminus and lysine side chain) of the peptides are the major targets for peptide acylation by PLGA.[9] Recently, there have been studies on octreotide, which is the active ingredient of Sandostatin LAR depot from Novartis Pharma (Basel, Switzerland).[10-13] Na et al suggested PEGylation, polyethylene glycol(PEG) conjugation, technology as a strategy for preventing the peptide acylation problem, in which mono-PEGylation at the N-terminus of octreotide could completely inhibit octreotide acylation by PLGA.[14,15] Accordingly, the peptide acylation is defined as the formation of peptide impurities resulting from the chemical reaction between the peptide and polymer in PLGA formulations, which may lead to changes in the biological properties of peptides such as the loss of biological activity or a change in immunogenicity. However, there are no reports of the biological properties of the acylated peptides as impurities.

This study investigated the acylation of growth hormone-releasing peptide-6 (GHRP-6) by PLGA and the biological activity of the acylated peptide. GHRP-6 is a synthetic hexapeptide (His-D-Trp–Ala–Trp-D-Phe-Lys-NH_2) that has been shown to effectively stimulate the release of growth hormone in animals and humans.[16] In order to examine the acylation pattern of GHRP-6, the formation of acylated GHRP-6 by different molecular weight (MW) PLGA polymers was monitored using reversed-phase high-performance liquid chromatography (RP-HPLC) and characterized by MALDI-TOF MS. In this study, the in vivo growth hormone-releasing activity of acylated GHRP-6 was examined in rats to determine

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the effect of peptide acylation by PLGA on the biological activity of GHRP-6.

Materials and Methods

Materials

The hydrophilic 50:50 PLGA 5020 (MW 20 000), PLGA 5010 (MW 10 000), and PLGA 5005 (MW 5000) were purchased from Wako Pure Chemical Ltd (Tokyo, Japan). The hydrophilic 50:50 PLGA Resomer RG 503H (MW 33 000) was supplied by Boehringer Ingelheim (Ingelheim, Germany). The GHRP-6 was obtained from Bachem (Torrence, CA). The rat growth hormone (rGH) [^{125}I] assay kit was obtained from Amersham Biosciences (Piscataway, NJ). The trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Sigma (St Louis, MO). The methanol and acetonitrile in high-performance liquid chromatography (HPLC) grade were supplied by J.T. Baker (Philipsburg, NJ). All other chemicals were of analytical grade and were obtained commercially.

Monitoring of Acylation of GHRP-6 by PLGA

The acylation of GHRP-6 was monitored by incubating GHRP-6 with different MW PLGA polymers, such as PLGA 5005, PLGA5010, PLGA 5020, and RG 503H. One hundred milligrams of each PLGA polymer was suspended in 100 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mg of GHRP-6. Each solution was divided into 100 μL in a 1.5-mL Eppendorf tube and incubated at 37°C for 15 days. At a predetermined sampling time, the tubes were removed and then analyzed by RP-HPLC and MALDI-TOF MS.

HPLC Analysis

HPLC analysis of the intact and acylated GHRP-6 peptides was performed with a Capcell-pak RP-18 column (250 mm × 4 mm, 5 μm; Shisheido, Tokyo, Japan). A gradient elution method was used with mobile phase A (0.1% [vol/vol] trifluoroacetic acid (TFA) in water) and mobile phase B (0.1% [vol/vol] TFA in acetonitrile). The gradient was 75:25 (A:B) to 60:40 (A:B) over 10 minutes, with a flow rate of 1.0 mL/min. The UV absorbance was monitored at 215 nm.

MALDI-TOF MS

MALDI-TOF MS was performed using a Voyager-RP Biospectrometry Workstation (Applied Biosystems, Cambridge, MA). The analysis was performed in linear mode, and the data for the 2-ns pulses of the 337-nm nitrogen laser were averaged for each spectrum. Linear and positive-ion TOF detection were performed with an acceleration voltage of 25 kV and a laser intensity of ~10% higher than the threshold. The grid and guide-wire voltages were chosen for each spectrum in order to achieve the optimal signal-to-noise ratio. The spectra were obtained by summing over 256 laser shots to obtain comparable conditions, and the data were smoothed with a 19-point Savitzky-Golay filter. A saturated solution of α-CHCA in 50% acetonitrile containing 0.1% TFA, as the final concentration, was used as the matrix solution.

Biological Activity of Intact and Acylated GHRP-6 Peptides

The in vivo biological activities of intact and acylated GHRP-6 were evaluated by monitoring the serum rat growth hormone (rGH) levels in male Sprague-Dawley rats (200-220 g, 6 weeks old, Hanlim Experimental Animal Inc, Seoul, Korea). The acylated GHRP-6 was obtained by reacting with RG 503H. The rats were maintained under constant environmental conditions (22°C ± 1°C; 50% ± 5% relative humidity) and were fasted overnight before the experiment. Anesthesia was induced by an intramuscular injection of pentobarbital. After the IV administration of the samples (30 μg/rat), the blood samples were collected at different times for 4 hours. The GH concentrations were determined using an rGH [^{125}I] radioimmunoassay (RIA) kit. Animal studies were performed according to the Principles of Laboratory Animal Care Guide by the National Institutes of Health (Bethesda, MD).

Results and Discussion

RP-HPLC and MALDI-TOF MS were used to characterize the formation of the acylated GHRP-6 by a reaction with PLGA. Figure 1 shows an HPLC chromatogram of GHRP-6 incubated with RG 503H in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 15 days. With the exception of the intact GHRP-6 peak (retention time 7.8 minutes), an additional peak was observed at a retention time of 9.3 minutes. The additional peak was collected and analyzed by MALDI-TOF MS, which had been effectively used to identify the acylated peptides both inside and outside the microspheres.[9,17] Figure 2 shows the mass spectrum with 5 peaks at *m/z* 930.72, 953.30, 968.36, 989.51, and 1002.89. The peaks were assigned to mono-glycoyl-conjugated GHRP-6 (*m/z* 930.72), sodium mono-glycoyl-conjugated GHRP-6 (*m/z* 953.30), sodium mono-lactoyl-conjugated GHRP-6 (*m/z* 968.36), diglycoyl-conjugated GHRP-6 (*m/z* 989.51), and glycoyl-lactoyl-conjugated GHRP-6 (*m/z* 1002.89). The *m/z* values are consistent with the structures of GHRP-6 modified with glycolic acid (+58), lactic acid (+72), glycolic-glycolic acid (+116), and glycolic-lactic acid (+130).[10] This result indicates that nucleophilic activity of GHRP-6 is more susceptible to glycolide units than lactide units. Figure 3 proposes the acylation reaction mechanism of GHRP-6 with PLGA.

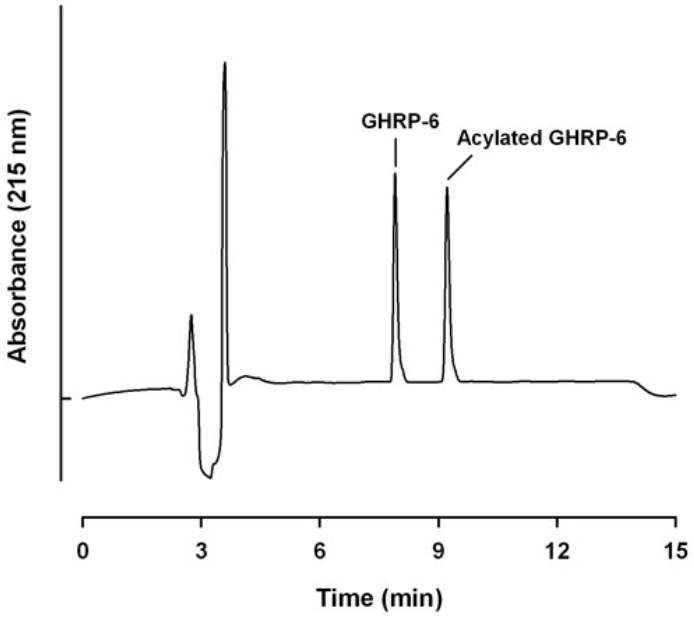

Figure 1. Reversed-phase high-performance liquid chromatogram of growth hormone-releasing peptide-6 (GHRP-6) incubated with poly(lactide-co-glycolide) (RG 503H) in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 15 days.

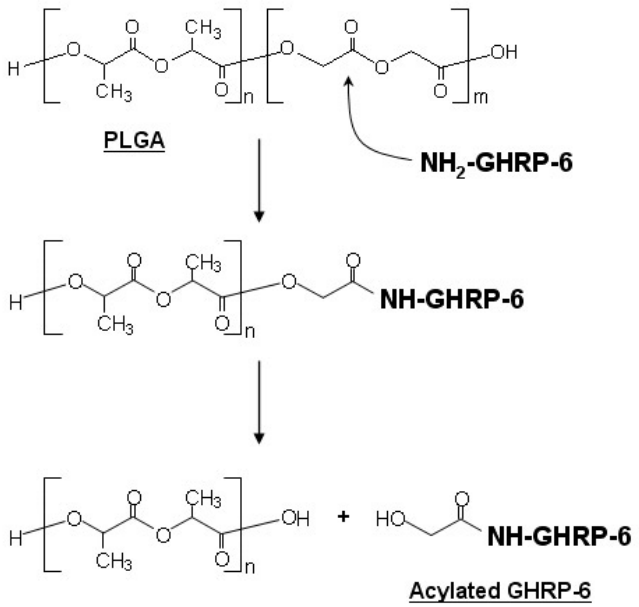

Figure 3. The proposed acylation reaction mechanism of growth hormone-releasing peptide-6 (GHRP-6) with poly(lactide-co-glycolide) (PLGA).

The level of acylated GHRP-6 produced by PLGA was monitored as a function of time (Figure 4). After incubating GHRP-6 with PLGA (Resomer RG 503H) in a 0.1 M sodium phosphate buffer of pH 7.4 at 37°C for 15 days, the mixture was centrifuged and the supernatant was analyzed by RP-HPLC. After 1 day, the GHRP-6 level remained below 10% of the initial amount in the supernatant. The amount was maintained for 4 days. This result indicates that GHRP-6 is rapidly adsorbed into the PLGA polymer through an ionic or hydrophobic interaction. Na and DeLuca reported that the initial adsorption of a peptide to hydrophilic PLGA is mainly due to an ionic interaction between the amino group of the peptide and the terminal carboxyl group of PLGA.[14]

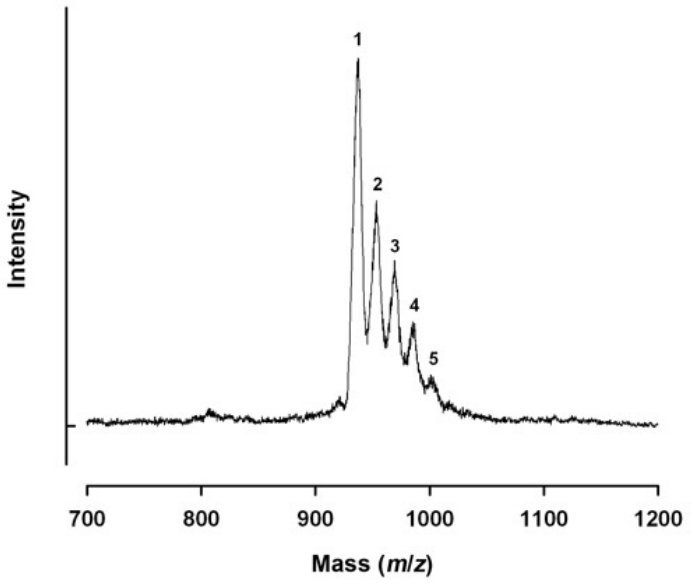

Figure 2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrum of the acylated growth hormone-releasing peptide-6 (GHRP-6) separated by reversed-phase high-performance liquid chromatography in Figure 1: (1) *m/z* 930.72 (GHRP-6 + glycolic acid); (2) *m/z* 953.30 (GHRP-6 + glycolic acid + Na^+); (3) *m/z* 968.36 (GHRP-6 + lactic acid + Na^+); (4) *m/z* 989.51 (GHRP-6 + 2 glycolic acid); and (5) *m/z* 1002.89 (GHRP-6 + glycolic acid + lactic acid).

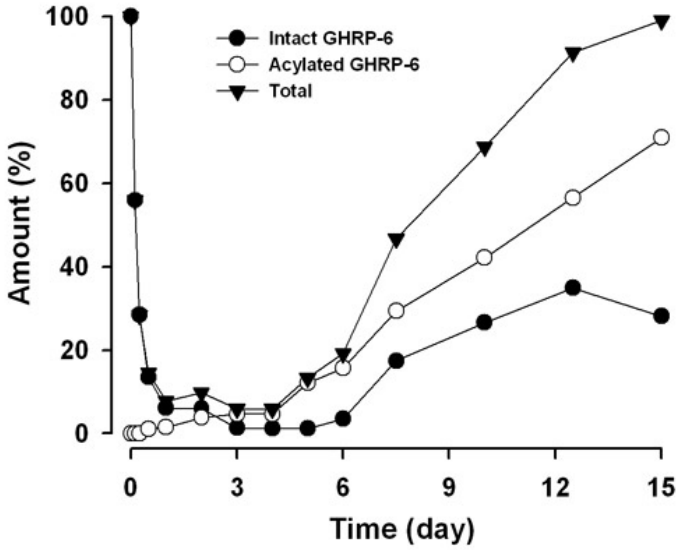

Figure 4. The production of acylated growth hormone-releasing peptide-6 (GHRP-6) after incubating GHRP-6 with poly(lactide-co-glycolide) (RG 503H) in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 15 days.

The acylated GHRP-6 was observed from day 2 and was gradually increased until day 4. The amount increased significantly from day 5 and reached up to 72% of the initial peptide amount by day 15. At that time, the total amount of the intact and acylated GHRP-6 peptides was almost 100%.

It is well known that the rate of PLGA degradation is dependent on its molecular weight.[18] The rate of degradation may be closely related to the degree of acylation of the peptide. The effect of the MW of PLGA on the acylation of GHRP-6 was examined with different MW 50:50 PLGA polymers of RG 503H (33 kd), PLGA 5020 (20 kd), PLGA 5010 (10 kd), and PLGA5005 (5 kd) (Figure 5). As shown in Figure 5A, the release of intact GHRP-6 following adsorption to PLGA was faster in the lower MW PLGA. After 15 days, the amount of intact GHRP-6 present in PLGA suspension was also higher with the lower MW PLGA. In the RG 503H PLGA suspension, the amount of intact GHRP-6 was significantly lower than that found in the other PLGA suspensions. However, the formation of acylated GHRP-6 was faster in the higher MW PLGA (Figure 5B). Although the amount of acylated GHRP-6 from RG503H was similar to the amount from the other polymers for the first 4 days, after 15 days, it was at least double that from other polymers. This phenomenon is believed to be related to the number of reaction target sites and the rate of PLGA degradation. In the same amount, a lower MW PLGA has fewer ester backbones in the PLGA, which is a target site for the nucleophilic attack of the reactive groups on the peptide, than the higher MW PLGA. The rate of degradation of the lower MW PLGA is generally faster than that of the higher MW PLGA. Figure 5C shows the pH profiles of the PLGA suspensions for the interaction study. The RG 503H PLGA suspension showed a higher pH level than the other PLGA suspensions. As peptide acylation favors a neutral pH over an acidic pH, the slower rate of RG 503H degradation may play a role in maintaining the conditions suitable for peptide acylation.

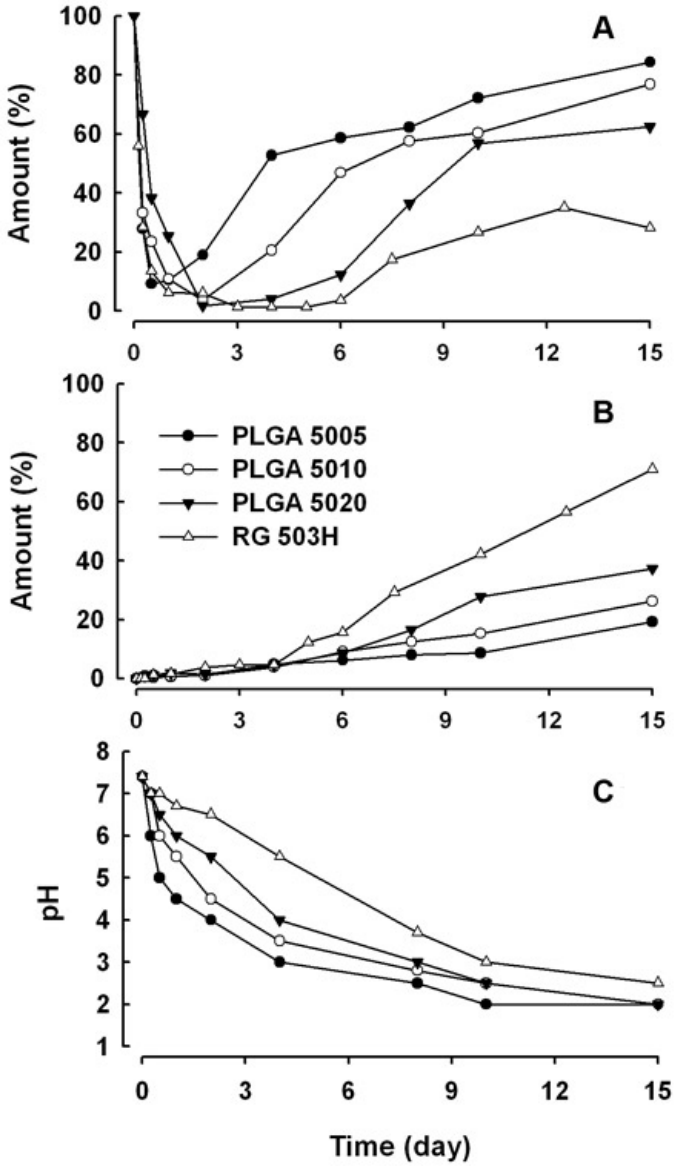

Figure 5. Effect of the molecular weight (MW) of poly(lactide-co-glycolide) (PLGA) on the acylation of growth hormone-releasing peptide-6 (GHRP-6) in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C. The profiles of (A) intact GHRP-6 and (B) acylated GHRP-6 present in PLGA suspensions; (C) pH profiles of different MW PLGA suspensions.

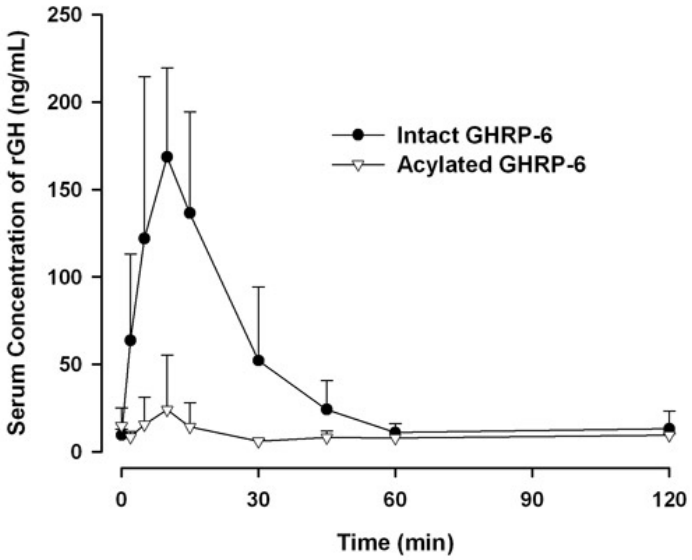

Figure 6. Mean rat growth hormone (rGH) releasing profiles obtained after the IV administration of the intact and acylated growth hormone-releasing peptide-6 (GHRP-6) peptides in rats. The values indicate the mean ± SD of 5 experiments.

The in vivo biological activities of acylated GHRP-6 as well as the intact GHRP-6 were evaluated by monitoring the released serum rGH levels after a single dose administration to rats (30 μg/rat) through an IV injection (Figure 6). The peak fractions collected from RP-HPLC corresponding to the

intact and acylated GHRP-6 peptides were administered to rats. After administering the intact GHRP-6, the peak value of rGH in rat serum (168.6 ng/mL) was found ~10 minutes later, and the value declined to basal level thereafter (~10 ng/mL) over a 60-minute period. The rGH release by the acylated GHRP-6 was much lower than that of the intact GHRP-6 group. At 10 minutes, the peak level reached 24.0 ng/mL and then declined to less than 10 ng/mL by 30 minutes. This result indicates that acylation significantly decreases the biological activity of GHRP-6.

CONCLUSION

HPLC and MALDI-TOF MS showed that the level of GHRP-6 acylation increased with increasing MW PLGA, which may be owing to the slower rate of polymer degradation. The acylated GHRP-6 showed much lower activity than the intact GHRP-6. This finding suggests that the acylation of GHRP-6 by PLGA decreases the effectiveness of the GHRP-6 formulation containing PLGA such as microspheres. This is the first report of the effect of peptide acylation on the biological activity. Overall, a strategy for the peptide stabilization such as PEGylation is needed to develop PLGA microspheres containing GHRP-6.

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REFERENCES

1. Gombotz WR, Pettit DK. Biodegradable polymers for protein and peptide drug delivery. *Bioconjug Chem.* 1995;6:332–351.

2. Sinha VR, Trehan A. Biodegradable microspheres for parenteral delivery. *Crit Rev Ther Drug Carrier Syst.* 2005;22:535–602.

3. O'Donnell PB, McGinity JW. Preparation of microspheres by the solvent evaporation technique. *Adv Drug Deliv Rev.* 1997;28:25–42.

4. DeLuca PP, Woo BW, Jiang G, Jo YW. Preparation and characterization of a composite PLGA and poly(acryloyl hydroxyethyl starch) microsphere system for protein delivery. *Pharm Res.* 2001;18:1600–1606.

5. Murty SB, Wei Q, Thanoo BC, DeLuca PP. In vivo release kinetics of octreotide acetate from experimental polymeric microsphere formulations using oil/water and oil/oil processes. *AAPS PharmSciTech.* 2004;5:E49.

6. Van de Weert M, Hennink WE, Jiskoot W. Protein instability in poly(lactic-co-glycolic acid) microparticles. *Pharm Res.* 2000;17:1159–1167.

7. Bilati U, Allemann E, Doelker E. Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. *Eur J Pharm Biopharm.* 2005;59:375–388.

8. Lucke A, Kiermaier J, Gopferich A. Peptide acylation by poly(alpha-hydroxy esters). *Pharm Res.* 2002;19:175–181.

9. Na DH, Youn YS, Lee SD, et al. Monitoring of peptide acylation inside degrading PLGA microspheres by capillary electrophoresis and MALDI-TOF mass spectrometry. *J Control Release.* 2003;92:291–299.

10. Na DH, Murty SB, Lee KC, Thanoo BC, DeLuca PP. Preparation and stability of PEGylated octreotide for application to microsphere delivery. *AAPS PharmSciTech.* 2003;4:E72.

11. Murty SB, Goodman J, Thanoo BC, DeLuca PP. Identification of chemically modified peptide from poly(D,L-lactide-co-glycolide) microspheres under in vitro release conditions. *AAPS PharmSciTech.* 2003;4:E50.

12. Murty SB, Thanoo BC, Wei Q, DeLuca PP. Impurity formation studies with peptide-loaded polymeric microspheres. I. In vivo evaluation. *Int J Pharm.* 2005;297:50–61.

13. Murty SB, Na DH, Thanoo BC, DeLuca PP. Impurity formation studies with peptide-loaded polymeric microspheres. II. In vitro evaluation. *Int J Pharm.* 2005;297:62–72.

14. Na DH, DeLuca PP. PEGylation of octreotide. I. Separation of positional isomers and stability against acylation by poly(D,L-lactide-co-glycolide). *Pharm Res.* 2005;22:736–742.

15. Na DH, Lee KC, DeLuca PP. PEGylation of octreotide. II. Effect of N-terminal mono-PEGylation on biological activity and pharmacokinetics. *Pharm Res.* 2005;22:743–749.

16. Camanni F, Ghigo E, Arvat E. Growth hormone-releasing peptides and their analogs. *Front Neuroendocrinol.* 1998;19:47–72.

17. Na DH, DeLuca PP, Lee KC. Direct determination of the peptide content in microspheres by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal Chem.* 2004;76:2669–2673.

18. Mehta RC, Thanoo BC, DeLuca PP. Peptide containing microspheres from low molecular weight and hydrophilic poly(d,l-lactide-co-glycolide). *J Control Release.* 1996;41:249–257.