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Preparation, Characterization, and In Vivo Evaluation of Salmon Calcitonin Microspheres

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ABSTRACT

Purpose. This study was done to prepare, characterize, and evaluate salmon calcitonin (sCT) microspheres (ms) in vivo using a low molecular weight, hydrophilic 50:50 poly (D,L-lactide-co-glycolide) polymer (PLGA). **Methods.** sCT ms were prepared by a dispersion/solvent extraction/evaporation process and characterized for drug content, particle size, surface morphology, and structural integrity of encapsulated peptide. Peptide stability and binding to the polymer was studied in 0.1 M phosphate buffer (PB), pH 7.4, and 0.1 M acetate buffer (AB), pH 4.0. Serum sCT levels were monitored for 2 weeks after subcutaneous injection of sCT ms to rats. **Results.** sCT ms were essentially free of discernible surface pores with a particle size distribution in the range of 16 to 89 mm and mean particle size of 51 and 53 mm for 2 batches. Fourier Transform Matrix-assisted Laser Desorption mass spectrometry of the extracted peptide showed that the encapsulation process did not alter its chemical structure. The peptide was substantially more stable in AB than in PB. Peptide binding to the polymer was dependent on pH and was markedly higher in PB than in AB. In vivo study proved that elevated serum sCT levels could be sustained for at least 10 days after administration of sCT ms to rats at a dose of 1.0 mg/kg. **Conclusions.** It was demonstrated that sCT could be incorporated into polymeric ms prepared from a low molecular weight, hydrophilic PLGA using a dispersion technique without altering molecular structure. A 2-week formulation was prepared at a dose of 1.0 mg/kg.

KEYWORDS: Salmon calcitzzonin (sCT); microspheres; PLGA; peptide interaction

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INTRODUCTION

Salmon calcitonin (sCT), a 32 amino acid polypeptide, has a physiological role in the regulation of calcium homeostasis and is a potent inhibitor of bone resorption. Results from clinical trials and animal studies have shown that sCT depresses bone turnover and prevents bone loss in early postmenopausal women [1-3] and ovariectomized rats [4-6]. sCT is being used clinically to treat osteoporosis. It is currently formulated as a sterile solution for intramuscular or subcutaneous injection or as a nasal spray. Being a polypeptide, sCT is vulnerable to digestive degradation and has a very short half-life in the body; $t^{1/2}$ after parenteral administration is approximately 15 to 20 minutes [7]. The treatment of osteoporosis requires long-term therapy, and because of these properties, which are drawbacks to long-term therapy, patient compliance can be severely limited, especially when a daily injection regimen of sCT is required. Hence, there is an ongoing effort to develop controlled-release dosage forms (formulations) that can effectively deliver sCT.

Poly (D,L lactide-co-glycolide) copolymer (PLGA) has shown promise for the delivery of sCT [8]. This copolymer is commercially available in a variety of comonomer ratios and molecular weights. Previous work in this laboratory has shown that sCT could be successfully encapsulated with high loading efficiency in PLGA microspheres (ms) made from a high molecular weight, hydrophobic polymer that had end groups protected (alkylated) [9]. However, wetting was difficult and required a long time for bond cleavage and biodegradation, and the release of sCT from these ms occurred in intermittent pulses through 120 days, followed by a large peak between 120 and 148 days. The latter coincided with complete solubilization of the polymer [unpublished work]. Recently, because of the interest in their application as

drug delivery matrices, PLGA polymers with hydrophilic end groups have become available. This study focused on preparing and characterizing a sustained-release delivery system for sCT made from a 50:50, low molecular weight, hydrophilic PLGA and evaluating the system in vivo.

MATERIALS AND METHODS

Materials

sCT was purchased from Bachem, Inc (Torrance, CA). PLGA RG502H polymer (lot # 34035, molecular weight [MW] 7800 d) was obtained from Boehringer Ingelheim, Inc (Ingelheim, Germany) and was used for ms preparation. Polyvinyl alcohol (PVA) (average MW 30 000-70 000) was obtained from Sigma Chemical Co (St Louis, MO). The solvents and other excipients were analytical grade and were purchased from commercial sources. Female Sprague Dawley rats that weighed approximately 250 g and were 90 days old were purchased from Harlan Laboratories (Indianapolis, IN), and the in vivo studies were conducted at the University of Kentucky College of Pharmacy Animal Research Facility in accordance with the institutional guidelines.

HPLC method for peptide assay

The peptide was analyzed by reverse-phase high-performance liquid chromatography (HPLC) using 2 LC-6A pumps, an SIL-6B autoinjector, an SPD-6AV detector, and an SCL-6B system controller (all from Shimadzu Scientific Instruments, Inc, Columbia, MD). The column used was a Bondclone 10, C-18 reversed-phase column (150 × 3.90 mm) with a Bondclone 10, C-18 (30 × 3.90 mm) guard column (Phenomenex, Torrance, CA). Gradient elution was accomplished with 0.1% trifluoroacetic acid in water (A) and acetonitrile, 0.1% trifluoroacetic acid (B) and increasing the amount of phase B from 30% to 50% over 10 minutes at a flow of 1.5 mL/min. Standard curves of sCT ranging from 6.25 to 200 µg/mL yielded linear responses over that concentration range with detection at 220 nm.

Polymer Characterization

The molecular weight distribution of RG502H PLGA polymer was determined by gel permeation chromatography (GPC) using a Waters M-45 solvent delivery system with a Waters 990 Photodiode Array Detector and ultrastyragel columns connected in series (7.8 × 300 mm each, one with 10^4 Å pores and one with 10^3 Å pores). The polymer was dissolved in tetrahydrofuran (THF) at 0.1% (wt/vol), eluted with THF at 1 mL/min and analyzed at 230 nm. Number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (M_w/M_n) were determined.

T_g was determined using a differential scanning calorimeter (DSC) equipped with a refrigeration system (Thermal Analysis Instruments DSC 2920, New Castle, DE). From 5 to 10 mg of the polymer was sealed in an aluminum pan. Then, under a nitrogen purge, the sample and reference (empty pan) were heated at a rate of 5ºC min^{-1} from 20ºC to 80ºC, cooled at 10ºC min^{-1} to 20ºC, then heated at 5ºC min^{-1} to 65ºC.

Preparation of sCT ms

sCT ms were prepared by a dispersion method followed by solvent extraction and evaporation. Briefly, a solution of peptide in methanol was combined with a solution of PLGA in methylene chloride and stirred until clear. The solution was then slowly injected into a 1 L reactor with baffles (Ace Glass Inc, Vineland, NJ) containing the continuous phase (CP) (0.35% [wt/vol] solution of PVA, pH 7.2) and stirred at 3500 rpm with a Silverson L4R homogenizer (Silverson Machines Ltd, Waterside, Chesham, Buckinghamshire, UK). The temperature of the reactor was maintained initially at 25ºC for 30 minutes and then at 35ºC for 3 hours. Once ms were formed and hardened, the contents of the reactor were transferred to a filtration apparatus equipped with a 0.8 µ membrane filter (Gelman Sciences, Ann Arbor, MI), and the recovered product was rinsed with water and either lyophilized or dried under reduced pressure for 48 hours at room temperature. Blank ms, without sCT, were prepared similarly.

Microsphere Characterization

Peptide content in ms was determined by HPLC after dissolving the ms in methylene chloride and extracting the peptide with 0.1 M acetate buffer (AB), pH 4.0. Fourier Transform Matrix-assisted Laser Desorption (FT-MALDI) mass spectrometry with dihydrobenzene as the matrix and nitrogen laser at 260° intensity was used to determine the molecular weight of sCT. The integrity of encapsulated sCT was confirmed by comparing the mass spectrum of sCT extracted from the ms to that of standard sCT solution.

The glass transition temperature of ms was determined by DSC. Particle size distribution was determined using a laser diffraction technique (Malvern 2600c Particle Sizer, Malvern, UK). Surface morphology was analyzed by scanning electron microscopy (SEM) with a Hitachi S 800 (Tokyo, Japan) instrument after palladium/gold coating on aluminum stubs. Total product yield was assessed gravimetrically on the basis of polymer/drug recovery.

IN VITRO STUDIES

Peptide Stability

Stock solutions of sCT were prepared by dissolving 10 mg of sCT in 50 mL of 0.1 M phosphate buffer (PB), pH 7.4, or 0.1 M AB, pH 4.0. All solutions were used immediately upon preparation. Peptide stability was determined in PB and AB at 3 temperatures: 4ºC, 25ºC, and 37°C. Approximately 5 mL of fresh stock solutions were placed in 7 mL glass scintillation vials, and the vials were sealed. Periodically, aliquots were removed from each vial, and the amount of intact peptide was assayed by HPLC.

Peptide Adsorption to PLGA ms

Stock solutions of sCT were prepared by dissolving 15 mg of sCT in 30 mL of 0.1 M PB, pH 7.4, or 0.1 M AB, pH 4.0. All solutions were used immediately upon preparation. One milliliter of sCT stock solutions in PB and AB were incubated at 37°C with 10 mg of blank ms. At each time point, 2 samples of each suspension were removed from the incubator. The samples were centrifuged, and the amount of peptide in the supernatant was determined by HPLC.

In Vivo Evaluation of sCT ms

sCT ms were evaluated in vivo in female Sprague-Dawley rats. Three groups of rats were subjected to the treatments described in Table 1. Free sCT was dissolved in 0.9% saline, and a single injection was administered subcutaneously at a dose of 71 μg/kg. The microsphere lots were suspended in a mixture of 1% CMC and 2% mannitol. The formulations were injected at point body weight volume (ie, μL administered = g body weight) subcutaneously, just below the neck region. A single injection was given to each animal immediately after collecting a predosing sample.

Blood samples were collected from the tail vein under light ether anesthesia. Samples from the animals receiving free sCT were collected at 0, 1, 6, and 24 hours after injection. Samples from the animals receiving the microsphere formulations at 0.5 mg/kg and 1.0 mg/kg doses were collected at 0, 1 hour, and 1, 3, 6, 9, 12, and 15 days after injection. The samples were centrifuged in Microtainer tubes (Becton Dickinson, Franklin Lakes, NJ) to separate and collect the serum. Serum samples were frozen and stored at -20°C until analysis using a radioimmunoassay (RIA).

Table 1. Treatment Regimens of Free sCT and sCT ms Administration in Rats

Group	Description	n	sCT Dose (mg/kg)
Group 1	Free sCT	4	0.071
Group 2	sCT ms (sCT02)	4	0.5
Group 3	sCT ms (sCT02)	4	1.0

sCT indicates salmon calcitonin.

Serum sCT analysis

Serum sCT was measured by ^{125}I RIA with a commercially available kit (Peninsula Laboratories, San Carlos, CA). Serum samples were incubated with rabbit sCT antiserum for 24 hours at 4°C followed by addition of ^{125}I-labeled sCT. After an additional 24-hour incubation, a second antibody-goat antirabbit immunoglobulin G-was added, followed by 90-minute incubation at room temperature to separate the antibody-bound and free sCT. The antibody-bound radioactivity was then measured by a MINAXI g-counter (Packard, Downers Grove, IL).

RESULTS

Polymer Characterization

The M_w and M_n of RG502H were approximately 7800 and 4500 d, respectively (polydispersity 1.8). RG502H showed a T_g of 36.4°C.

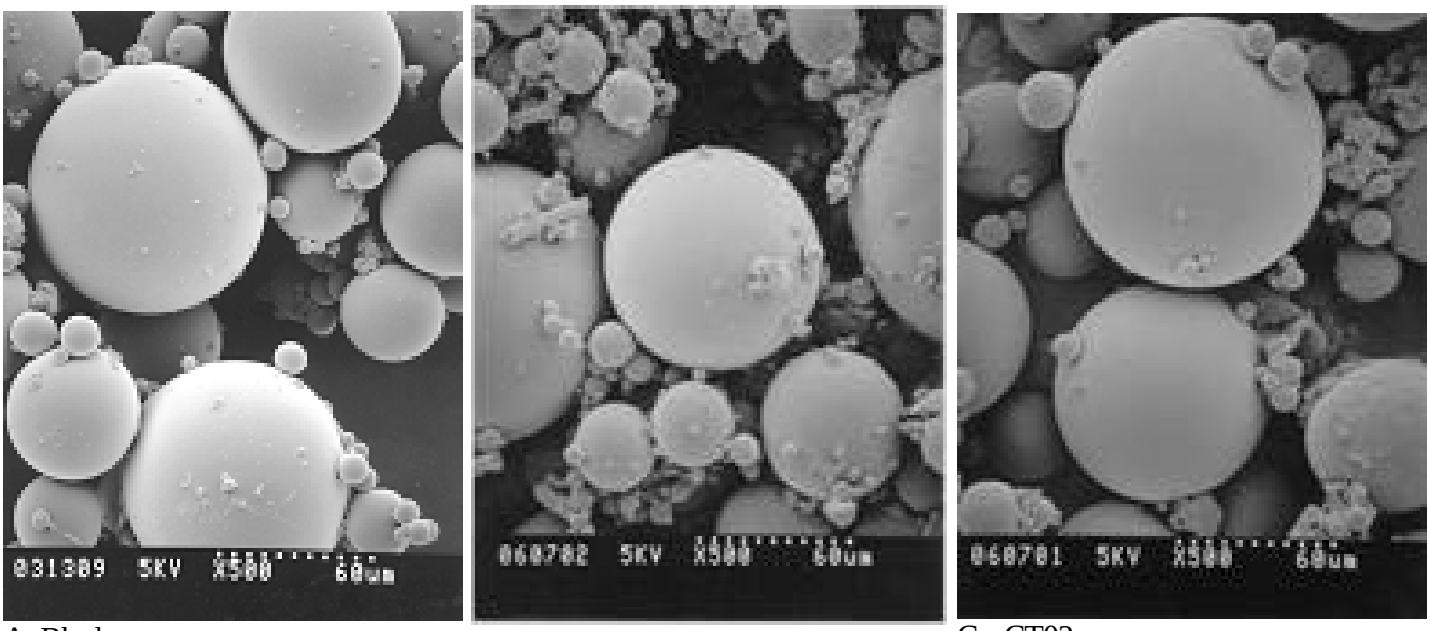
Microsphere Characterization

Thermal analysis of blank ms prepared from RG502H polymer showed an increase in T_g (39.5°C) compared to that of the raw polymer. However, the T_g of the 2 lots of sCT ms, sCT01 and sCT02 (40.5°C and 40.3°C, respectively), was not much different than that of the blank ms (Table 2).

Table 2. Physical Properties of sCT and Blank ms

Batch	sCT Load (wt %)	Incorporation Efficiency (wt %)	Yield (wt %)	Tg (°C)	Particle Size (μm)		
					< 90%	< 50 %	< 10 %
Blank ms	**0**	**-**	**80**	**39.5**	**86**	**48**	**18**
sCT ms(sCT01)	**5.1**	**102**	**78**	**40.5**	**89**	**53**	**17**
sCT ms(sCT02)	**4.5**	**90**	**80**	**40.3**	**89**	**51**	**16**

sCT indicates salmon calcitonin; ms, microspheres.



A. Blank ms B. sCT01 ms C. sCT02 ms

Figure 1. Scanning electron micrographs showing the surface morphology of blank and salmon calcitonin (sCT)

The average particle size of the 2 lots of sCT ms was 53 (sCT01) and 51 (sCT02) mm. Incorporation efficiencies of 102% and 90% revealed that most of the sCT had been encapsulated within the ms. Examination of the surface morphology by SEM revealed that both sCT as well as blank ms were free of discernible surface pores (Figure 1). The mass spectrum of sCT extracted from ms (Figure 3) was similar to that of a standard sCT solution (Figure 2)**[[figures should be called out in numerical order; rewrite sentence or renumber figures?]]**, which confirmed that the structural integrity of the peptide was intact within the ms.

Peptide Stability

Figure 4 shows the effect of pH and temperature on the stability of sCT in solution. The peptide was substantially more stable in AB, pH 4.0, than in PB, pH 7.4. In PB at 37°C, 37% of the peptide was degraded within 24 hours and 100% after 3 days. In AB at 37°C, 95% of the initial amount still remained after 3 days, 90% after 6 days, 71% after 15 days, and 63% after 22 days. Peptide degradation was slower at lower temperatures in both buffers, and as much as 82% and 91% of the initial amount still remained after 22 days at 22°C and 4°C, respectively, in AB.

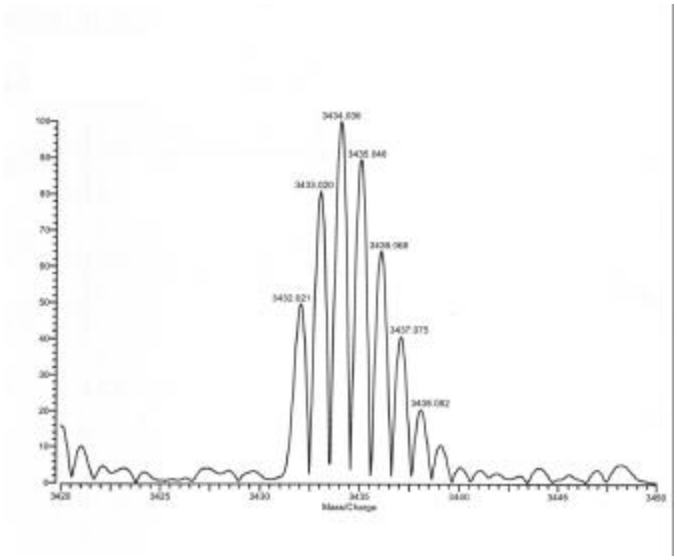


Figure 2. Fourier Transform Matrix-assisted Laser Desorption (FT-MALDI) mass spectrum of salmon calcitonin (sCT) standard in 0.1 M acetate buffer.

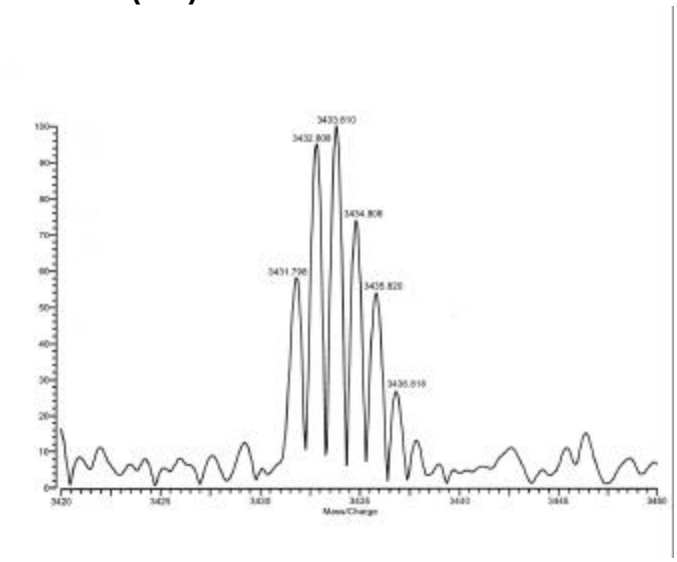


Figure 3. Fourier Transform Matrix-assisted Laser Desorption (FT-MALDI) mass spectrum of sCT extracted from salmon calcitonin (sCT) microspheres (ms) in 0.1 M acetate buffer.

Peptide Adsorption to PLGA ms

As shown in Figure 5, there was not much adsorption of sCT to blank PLGA ms in 0.1 M AB, pH 4.0, at 37°C, and the amount of peptide remaining in the supernatant throughout 24 hours was 97% ± 2% of the initial amount. In contrast, the amount of peptide adsorbed to the blank ms in 0.1 M PB, pH 7.4, increased with time, and approximately 19.7 μg of peptide was adsorbed to 1 mg of ms after 5 hours (41% of the initial amount). Although no samples were analyzed between 6 and 24 hours, saturation binding appeared to occur within 24 hours at approximately 20 µg/mg.

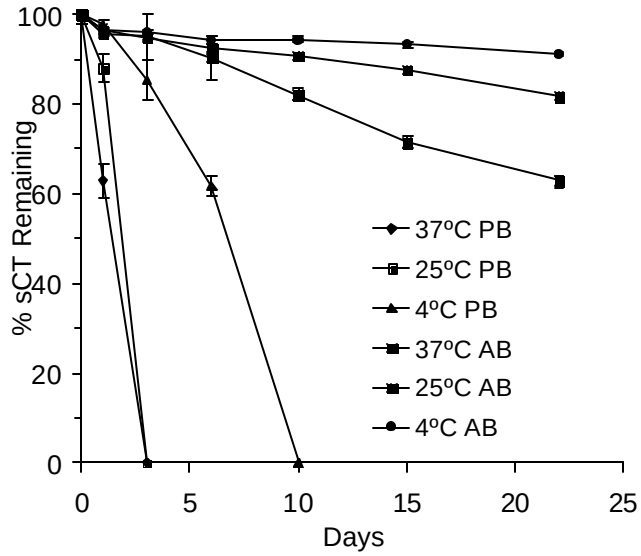


Figure 4. Stability of salmon calcitonin (sCT) at 37ºC, 25ºC, and 4ºC in 0.1M phosphate buffer (PB) and acetate buffer (AB).

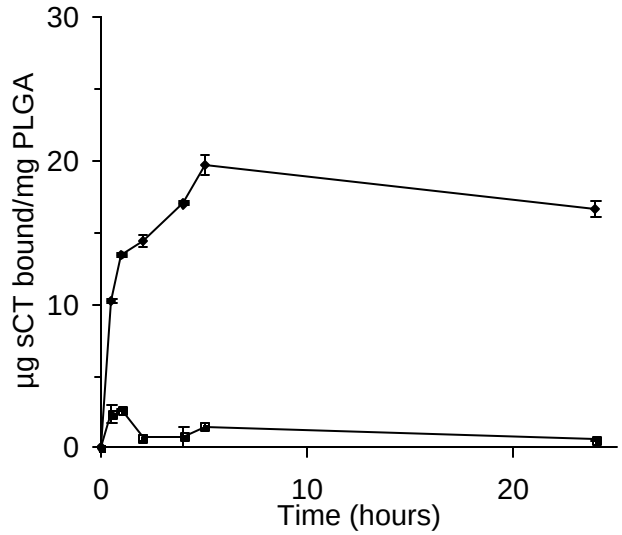


Figure 5. Adsorption of salmon calcitonin (sCT) to blank ms in 0.1 M phosphate (PB) and acetate (AB) buffers.

In Vivo Study

Single administration of free sCT to rats at 71 μg/kg dose (Figure 6A) resulted in a serum sCT peak 1 hour later (observed C_{max} 760 pg/mL). As expected, the sCT levels returned rapidly to baseline levels at 24 hours, since sCT has a very short half-life in vivo after parenteral administration [7]. Administration of 0.5 mg/kg dose of sCT microspheres (Figure 6B) resulted in a C_{max} of 791 pg/mL at 1 hour. Elevated serum sCT levels were sustained for at least 6 days, and

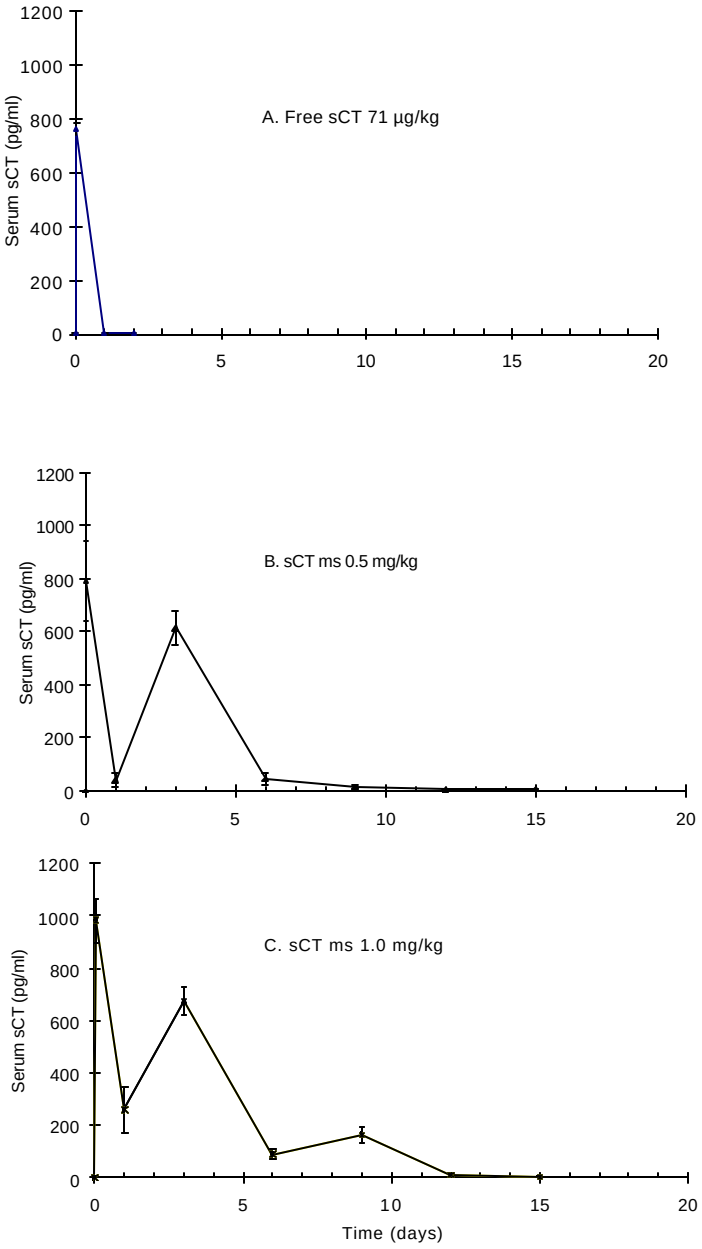


Figure 6. Serum sCT levels after administration of free sCT and sCT ms to rats (n = 4).

detectable levels were observed for 9 days following administration. Administration of 1.0 mg/kg dose of sCT microspheres produced a C_{max} of 984 pg/ml at 1 hour (Figure 6C), and elevated serum sCT levels were sustained beyond 9 days after administration, with the levels returning to baseline after 12 days.

DISCUSSION

GPC analysis of the raw 502H polymer showed that the M_w was 7800 d. This was lower than the typical 502H lots, which have M_w in the range of 10 000 d. This would account for the shorter duration of elevated serum levels than desired. DSC measurements indicated that the blank ms prepared from 502H polymer had a slightly higher T_g (39.5°C) than did the raw polymer (36.4°C). This slight shift in T_g was most likely the result of the processing of the polymer during the microsphere preparation process. On the other hand, T_g of sCT ms (40.3°C and 40.5°C) was comparable to blank ms. A marked shift in T_g would have indicated a strong interaction between sCT and the polymer (in the dry state). Thus, the DSC results suggest that in the dry state, sCT did not interact very strongly with the hydrophilic, low molecular weight 502H PLGA polymer. A higher molecular weight 502H polymer would have a slightly higher T_g, which would facilitate processing.

FT-MALDI mass spectrum of the extracted peptide showed that the encapsulation process did not alter its chemical structure. Stability studies confirmed that the peptide was substantially more stable in solution at a pH of 4.0 than at a pH of 7.4, which was consistent with previous studies that had reported a pH of 3.3 for maximum stability [10]. There was very little adsorption of sCT to blank PLGA ms at a pH of 4.0. However, the adsorption increased dramatically at a pH of 7.4. These results indicate that the nonspecific adsorption of sCT to PLGA ms was greater at a pH closer to the isoelectric point (pI) of sCT, which is around 10.2 [11]. This is consistent with earlier work on sCT adsorption to a higher molecular weight PLGA (M_W 34 000) containing alkylated (hydrophobic) end groups, where maximum adsorption occurred near the pI of sCT and almost no adsorption was observed at a pH less than 6 [11]. It was thought that a higher net charge on the peptide molecules, at a pH below pI, could result in repulsion within and between peptide molecules. At the pI of the peptide, molecules with a zero net charge could approach each other more closely and form a more compact conformation, resulting in more effective adsorption. The results of that study suggested that hydrophobic interactions played an important role in the adsorption. Interestingly, at pH 7.4, the amount of sCT adsorbed to the low molecular weight (hydrophilic) PLGA (502H) was much lower (19.7 µg

sCT/mg PLGA) compared with the high molecular weight (hydrophobic) PLGA (>80 µg sCT/mg PLGA) [11]. Because elevated serum sCT levels could be sustained for about 10 days following administration of sCT ms to rats at a dose of 1.0 mg/kg, this was thought to be promising as a 2-week formulation, especially with a higher molecular weight 502H PLGA.

CONCLUSIONS

It was demonstrated that sCT could be incorporated into polymeric ms prepared from a low molecular weight, hydrophilic PLGA with the dispersion technique without altering its molecular structure. DSC measurements indicated a lack of strong interaction between sCT and the polymer in the dry state. Peptide stability was greater at lower pH, while binding was significantly higher at higher pH. Elevated serum sCT levels could be sustained for at least 10 days following administration of 1.0 mg/kg of sCT ms to rats.

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