Temperature and pH-sensitive Polymers for Human Calcitonin Delivery

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Purpose. Stimuli-sensitive polymers are suitable candidates for oral peptide drug delivery vehicles since they will prevent gastric degradation in the stomach while providing a controlled release of a peptide drug such as calcitonin later. The purpose of this study was to fabricate polymeric beads from pH/temperature sensitive linear terpolymers (poly(N-isopropylacrylamide-co-butylmethacrylate-co-acrylic acid)) and load them with a peptide drug, human calcitonin, which was dissolved in aqueous phase.

Methods. The polymeric beads were formed by solubilizing a cold, aqueous solution of temperature sensitive polymer with human calcitonin. This solution was added dropwise into an oil bath kept at a temperature above the LCST of a polymer, precipitating polymer and entrapping the peptide. The quantity and the physical state of the peptide were analyzed by reverse-phase HPLC, CD and FTIR and its biological activity after loading was determined in vivo.

Results. The loading efficiency and stability of human calcitonin into the polymeric beads was studied as a function of pH and ionic strength of the loading buffer and temperature of the oil bath. Final optimal loading conditions were 20 mM glycine/HCl buffer, pH 3.0 containing 0.15 M NaCl as a dissolution medium and 23°C as the oil bath temperature. Loading and release of human calcitonin were also studied as a function of acrylic acid content in the terpolymers. As the acrylic acid content increased from 0 to 10 mol %, the loading efficiency and stability of calcitonin improved significantly. The same trend was observed for the quantity of released calcitonin. In vivo biological activity of the released hormone was preserved.

Conclusions. The results showed that the beads made of the polymers with high content of acrylic acid (most hydrophilic) provided better loading, stability and release of human calcitonin. The designed beads represent a new potential system for oral delivery of calcitonin and other peptides.

KEY WORDS: human calcitonin; pH/temperature sensitive polymer; N-isopropylacrylamide copolymer; beads; calcitonin delivery.

INTRODUCTION

Human calcitonin (hCT) is a polypeptide consisting of 32 amino acids with a molecular weight of 3418 g/mol. This hormone is involved in the complex regulation of blood calcium level by inhibiting bone resorption. Calcitonin is therapeutically used for the treatment of bone diseases (osteoporosis, Paget's disease) and hypercalcemias of different origin. Currently, multiple parenteral injections of hCT are the most commonly used, due to the short half-life of hCT. Moreover, patients often suffer from severe side effects manifested particularly by sudden flush and gastrointestinal disorders (1).

In order to improve calcitonin therapy, alternative administration routes such as colonic (2), uterine (3), ocular (4), pulmonary (5), vaginal (6) and rectal (7,8) have been explored. Among alternative administration routes, intranasal administration is the most developed (9) and calcitonin sprays are becoming commercially available.

Many studies have also been focused on calcitonin delivery systems which serve to protect and stabilize the drug and release it in a controlled way. In particular, subcutaneous and oral delivery systems have been investigated (10–12). For example, polyisobutylcyanoacrylate nanocapsules were loaded with hCT and these nanocapsules protected the protein against proteolytic enzymes (13). Ariën et al. (14) designed calcitonin-loaded liposomes which were stable in acidic medium. Some barriers, such as low intestinal absorption, have not been overcome, hindering the efficiency of these new systems.

In this study, pH/thermosensitive polymeric beads were prepared using statistical terpolymer of N-isopropylacrylamide, butylmethacrylate (hydrophobic) and acrylic acid (hydrophilic) (15–17) and investigated as an oral drug carrier system. These polymers have recently attracted interest of many researchers because of their "intelligent" ability to deliver the drug they contain to desired places under optimal conditions (18, 19). Generally, these stimuli sensitive polymers possess the ability to load peptide drug in aqueous solution during the process of bead preparation. Such polymers are physically characterized by their lower critical solution temperature (LCST) or cloud point. The polymer is soluble below its LCST and precipitates above its LCST. Furthermore, the LCST is dependent on pH due to the ionization properties of acrylic acid (20, 21). The pH and temperature sensitive properties of this novel polymer prevent solubilization of the beads at acidic pH and 37°C and thus protect the drug from gastric degradation. The polymer becomes soluble around neutral pH and can release the drug in the intestinal tract. This concept has been demonstrated for insulin delivery (22). However, to make oral polypeptide delivery feasible, the absorption barrier of the intestinal wall has to be still overcome, usually through the use of enhancers, as demonstrated for insulin (23) and calcitonin (24) intestinal absorption.

In this paper, studies on the oral delivery system of human calcitonin based on pH/thermosensitive polymeric beads are reported. The optimal conditions of calcitonin loading into the beads in order to obtain a high loading efficiency and to preserve the bioactivity of the drug were studied. The structure, conformation and bioactivity of recovered calcitonin after loading and release were also analyzed. Future research will focus on an efficient enhancer and stabilizer that may be included into the polymeric bead system to overcome human calcitonin intestinal absorption barrier.

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Abbreviations: hCT—human calcitonin, AIBN—2,2′-azobis-isobutylonitrile, NiPAAm—N-isopropylacrylamide, BMA—butylmethacrylate, AA—acrylic acid, LCST—lower critical solution temperature, HPLC—high performance liquid chromatography, FTIR—Fourier transform infrared spectroscopy, CD—circular dichroism.

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MATERIALS AND METHODS

Materials

N-isopropylacrylamide, obtained from Eastman Kodak Company (Rochester, NY), was recrystallized from hexane. Acrylic acid, obtained from Aldrich Chemical Company (Milwaukee, WI), was purified by vacuum distillation at 39°C/10 mm Hg. Butylmethacrylate was obtained from Polysciences Inc. (Warrington, PA) and purified by vacuum distillation at 57°C/17 mm Hg. 2,2'-Azobis-isobutylonitrile, purchased from Eastman Kodak Company (Rochester, NY), was recrystallized from methanol. Heavy white mineral oil and decane were purchased from Aldrich Chemical Company (Milwaukee, WI). Acetonitrile, HPLC grade, was purchased from Fisher Scientific Inc. (Fair Lawn, NJ). Human calcitonin was kindly provided by Suntory Ltd (Tokyo, Japan). Calcium assay was purchased from Sigma Diagnostics (St. Louis, MO).

Polymer Synthesis and Characterization

The synthesis of linear terpolymers composed of NiPAAm, BMA and AA with varying feed ratio (NiPAAm/BMA/AA mol ratio = 90/10/0, 89/10/1, 88/10/2, 87/10/3, 85/10/5, 83/10/7 and 80/10/10) was carried out in dioxane with AIBN as a free radical initiator (7.41 mmol AIBN per mol monomer). Dried N_2 gas was bubbled through the solution for 20 min. to remove dissolved oxygen. The solution was polymerized overnight at 60°C under N_2 atmosphere (16 hours). The synthesized terpolymers were recovered by precipitation in n-hexane and purified by dissolving in tetrahydrofuran and reprecipitation in diethyl ether. The polymers were filtered and dried in vacuum overnight. It has been shown previously that under described conditions of polymerization, the feed monomers ratio corresponds to the actual copolymer composition (21). The molecular weights of terpolymers were determined by gel permeation chromatography as described earlier (22).

LCST determination. The LCSTs of the various polymers were determined by cloud point measurement. The polymer solutions (1% w/v) were prepared in 10 mM phosphate buffer pH 7.4, 0.15 M NaCl or 10 mM acetate buffer pH 4.5, 0.15 M NaCl or 20 mM glycine/HCl buffer pH 2.0, 0.15 M NaCl. The temperature of the solution was raised from 10°C to 80°C in 2°C increments every 10 min and the absorbance at 450 nm was measured using a Perkin-Elmer Lambda 7 UV/Vis spectrophotometer. The LCST was defined as the temperature at the inflection point in the absorbance versus temperature curve.

Bead Preparation

Calcitonin loaded beads were prepared from an aqueous solution (20 mM glycine buffer pH 3, 0.15 M NaCl) containing pH/temperature sensitive polymer (10% w/v) and calcitonin (0.2% w/v). The solution was kept at 4°C overnight to allow the solubilization of the polymer. The calcitonin/polymer solution (1 ml) at 4°C was added dropwise using a syringe and 25G needle into 50 ml mineral oil kept at a temperature above the LCST of the polymer (23°C or 32°C). The mineral oil was covered with 5 ml decane to reduce surface tension and to aid in the penetration of the added polymer solution. The formed beads were immediately washed with hexane, filtered and dried at room temperature in a rotary evaporator with aspiration. The beads had a spherical shape with an average diameter of 1.3 mm determined by optical microscopy. All the beads examined had diameters in the range from 0.8 to 1.6 mm.

Human Calcitonin Assay

The concentration of calcitonin solution was determined using reversed phase high performance liquid chromatography (HPLC). HPLC apparatus consisted of a Waters gradient system (automated gradient controller—model 680 and HPLC pump—model 501, Waters Chromatography division, Milford, MA). The samples were injected via an intelligent sample processor (WISP—model 712, Waters) connected to a UV detector (model 484, Waters) and an integrator (model 745, Waters). The C_4 column (5 μm, 4.6 × 250 mm, Vydac, Hesperia, CA) was equilibrated with 60% eluent A (10% acetonitrile, 0.1% trifluoroacetic acid) and 40% eluent B (60% acetonitrile, 0.095% trifluoroacetic acid). Calcitonin was eluted with a gradient starting at 60% eluent A and 40% eluent B to a mixture of 40% eluent A and 60% eluent B over 15 min at a flow rate of 1 ml/min. The absorbance of the eluent was recorded at 214 nm. The column was calibrated with hCT solutions of known concentration.

Loading Efficiency

The content of calcitonin in the beads was determined after complete dissolution of the beads at low temperature. The dried beads (about 100 mg) containing calcitonin were dissolved in 3 ml of 20 mM glycine/HCl buffer pH 3, 0.15 M NaCl at 4°C overnight. The resulting solution was heated up to 50°C for 10 min. After the polymer precipitated, the concentration of calcitonin in the supernatant was measured by HPLC assay. The amount of loaded calcitonin was independently determined by amino acid analysis.

Release Experiments

Calcitonin-loaded beads were placed in 10 ml isotonic PBS pH 7.4 at 37°C or in 10 ml 10 mM acetate buffer pH 4.5, 0.15 M NaCl at 15°C. At different times, 100 μl of the release medium was collected and replaced by the same volume of buffer. The collected sample was injected into reversed phase column and the concentration of released calcitonin determined.

Physical Stability of Human Calcitonin

The precipitation (fibrillation) of hCT solutions was followed by periodical visual examinations. This is possible due to the fact that kinetics of hCT aggregation is a nucleation-dependent phenomenon (25).

The conformation of recovered calcitonin after loading (see above) was analyzed by CD and FTIR. CD absorption spectra were obtained with a Jasco J-720 spectropolarimeter (Japan). Calcitonin recovered from polymeric beads and the freshly prepared calcitonin solution (0.5 mg/ml) in 20 mM glycine/HCl buffer pH 3, 0.15 M NaCl were placed in a cylindrical quartz cell (0.1 cm optical path length). The ellipticity (mdeg) was measured over 250 to 190 nm region.

For FTIR spectroscopy, hCT was recovered from the beads as described above for loading efficiency determination. However, D_2O was used instead of H_2O in order to avoid strong absorption band of OH. 20 mM glycine/DCl buffer pH 3, 0.15 M NaCl in D_2O was used. Spectra (2 cm^{-1} resolution, 64 scans, noise level 0.1, sensitivity 2, zero fill factor 4 and triangular apodization) were obtained over 4000 to 900 cm^{-1} region with a Digilab FTS 20/80 Fourier transform infrared spectrometer equipped with a liquid nitrogen cooled, narrow band MCT detector. The samples were placed between two calcium fluoride discs using 0.15 mm teflon spacers.

Bioactivity

The biological activity of hCT after loading (pH 3.0, 4°C, overnight) and release (pH 7.4, 37°C, 1 hour) was determined in a rate model (male Sprague-Dawley rats, 250 ± 50 g). The pH of hCT recovered solution after loading and polymer precipitation was adjusted to neutrality. Released hCT at pH 7.4, 37°C was used as such. As a reference, a fresh hCT solution was prepared in isotonic PBS pH 7.4. The samples, after appropriate dilution, were injected intramuscularly (10 μg/kg) and blood samples (100 μl) were collected before injection and 30, 60, 90, 120, 180, 240 and 300 min after injection. Immediately, 1 μl (5 U) of heparin (Elkins-Sinn, Cherry Hill, NJ) was added, the samples were centrifuged (10,000 rpm, 4°C) and the plasma separated. The plasma calcium levels versus time were obtained using quantitative commercial colorimetric calcium assay.

RESULTS AND DISCUSSION

Polymer Synthesis and Characterization

The temperature-sensitive homopolymer of N-isopropylacrylamide has an LCST of 31°C. In this study, NiPAAm was copolymerized with butylmethacrylate and acrylic acid. In general, the incorporation of a hydrophobic comonomer such as BMA increases the mechanical stability of the copolymer and decreases the LCST while incorporation of pH-sensitive and hydrophilic comonomer such as AA increases the LCST (21). The LCSTs of linear terpolymers poly(NiPAAm-co-BMA-co-AA) with constant 10 mol % BMA content and variable mol % ratio of NiPAAm and AA were determined at three different pH: 2.0, 4.5 and 7.4 (Fig. 1). At pH 2.0 and pH 4.5, the LCST is constant for the different copolymers. At pH 7.4, the LCST increases with AA content since AA is fully ionized thus significantly increasing overall hydrophilicity of the polymers (21, 22).

Preparation of hCT Loaded Beads

Because of the physical instability of hCT, the loading conditions in the polymer were studied to preserve the stability of the peptide and to obtain high loading efficiency into the beads. Different parameters governing the physicochemical properties of the polymer and the calcitonin were considered: pH, temperature, ionic strength and concentration. The loading conditions for calcitonin were initially adjusted according to results previously reported for insulin (22). The loading conditions were at acidic pH and low temperature (20 mM glycine/HCl buffer pH 3, 4°C) allowing the solubilization/swelling of the polymer independent of its composition. More importantly,

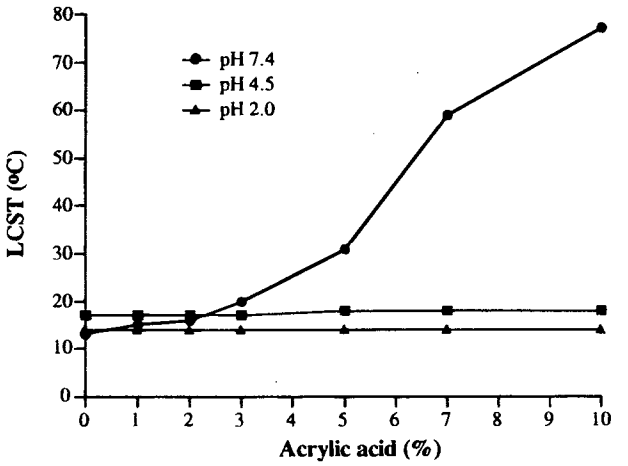

Fig. 1. Effect of acrylic acid content (in mol % of the feed composition) on the LCST of 1 % solutions of poly(NiPAAm-co-BMA-co-AA) at different pH. BMA content was kept constant at 10 mol %. See Experimental section for details. Some of the data has been already published (22).

under these conditions, the calcitonin is sufficiently stable. The ionic strength of the loading medium had to be analyzed in more details. It was necessary to have sufficiently high ionic strength to ensure formation of good quality beads. Polymer composition did not have a significant impact on bead quality. On the other hand, hCT fibrillation occurs within several hours at 23°C if the ionic strength is over 0.25 M (Fig. 2). Finally, 20 mM glycine/HCl buffer pH 3, 0.15 M NaCl at 4°C was chosen as the optimal loading medium.

The stability of calcitonin was also studied as a function of its concentration in the loading buffer. As expected, according to the phenomenon described by Arvinte et al. (25), the stability of human calcitonin was inversely proportional to its concentration (not shown). As a compromise, the concentration of 2 mg/ml of calcitonin was used throughout the study. Under these

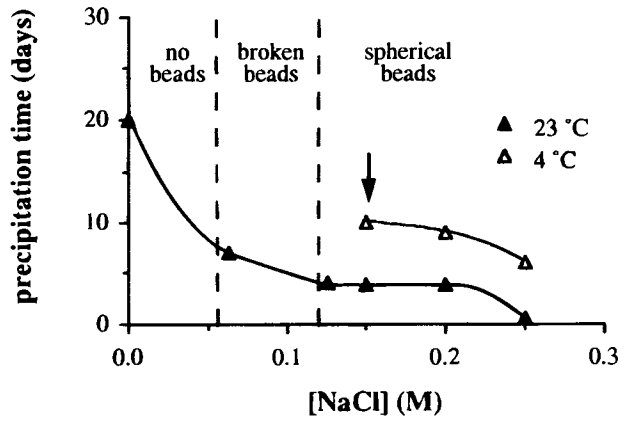

Fig. 2. Effect of the ionic strength of the loading buffer (20 mM glycine/HCl buffer, pH 3.0) on hCT solution stability (2 mg/ml) and quality of the beads (polymer composition 83/10/7 in mol %). HCT precipitation/fibrillation was followed visually and examined at two different temperatures (see insert). Precipitation time is defined as the moment when hCT starts to precipitate or fibrillate.

final loading conditions, hCT was stable over a period of 3 to 5 days at room temperature and a period of 8 to 10 days at 4°C.

The last parameter studied was the oil bath temperature into which the polymer/calcitonin solution was added dropwise. The oil bath was equilibrated at 32°C or 23°C and the loading efficiency for the beads formed was determined by HPLC. Increased loading efficiency was achieved with the colder oil bath. Around 50% of calcitonin was loaded into the beads at 23°C, rather than 35% loading at a temperature of 32°C for the most hydrophilic polymer containing 10 mol % of acrylic acid. Similar trend was observed for other polymers investigated. The increased oil bath temperature may lead to excessive bead shrinkage. During shrinkage, part of the hCT solution is ejected from the beads and so, the loading yield of calcitonin is decreased. Consequently, oil bath temperature of 23°C was used for the preparation of the beads.

The loading efficiency was studied for polymers with varying content of acrylic acid. The calcitonin amount in the beads was determined by two independent methods, HPLC and amino acid analysis. The results are listed in Table 1. Similar values were obtained by both methods. This demonstrates that after dissolution of beads and precipitation of polymer, the majority of loaded calcitonin is found in the supernatant. It is evident that polymers with high acrylic acid content (7 and 10 %) improved the loading efficiency when compared to polymers with low acrylic acid content. In other words, as polymer hydrophilicity decreases, loading efficiency into beads deteriorates.

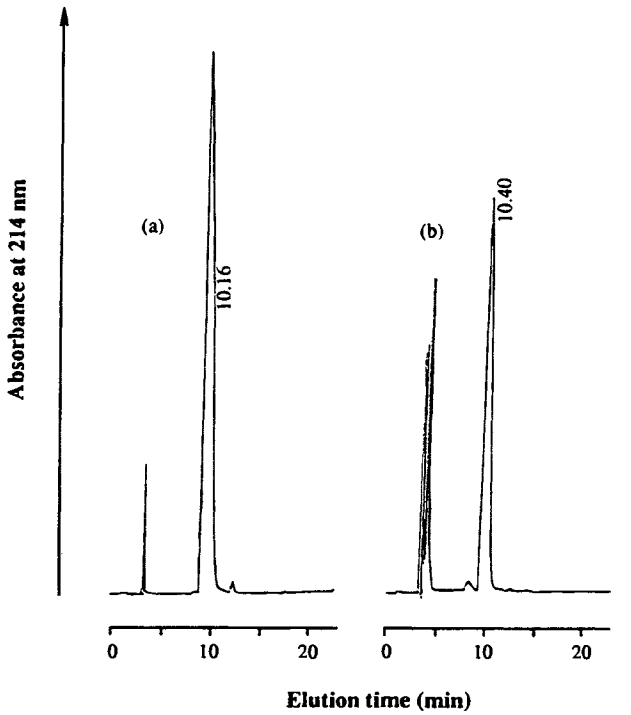

Fig. 3. RP HPLC elution profiles of hCT reference solution (0.5 mg/ml) (a) and hCT collected after loading in beads (b) (NiPAAm/BMA/AA mol ratio = 80/10/10). Injected volume was 200 µl.

Physical Stability of hCT

The physical state of the loaded calcitonin was also analyzed. Reverse phase HPLC chromatograms demonstrated that calcitonin was not degraded nor aggregated after contact with the polymer. Indeed, the retention times and the shapes of the peaks of freshly made hCT solution and loaded hCT are nearly identical (Fig. 3). Moreover, the far-UV CD spectra showed that no significant change in the conformation occurred during the loading (Fig. 4). However, a shift of the amide I band of the loaded hCT was observed by FTIR (Fig. 5). This phenomenon also occurred progressively within first 24 hours even after simple dissolution of hCT in 20 mM glycine/DCl buffer, pH 3, 0.15 M NaCl made in D_2O (Fig. 5). This shift has already been observed and assigned as an indication of fibrillation (25). According to HPLC, CD and visual inspection results of hCT solutions, no fibrillation occurred during first 24 hours. It is possible that the more sensitive FTIR analysis detected an initial reversible stage of fibrillation, not detectable by either CD or HPLC.

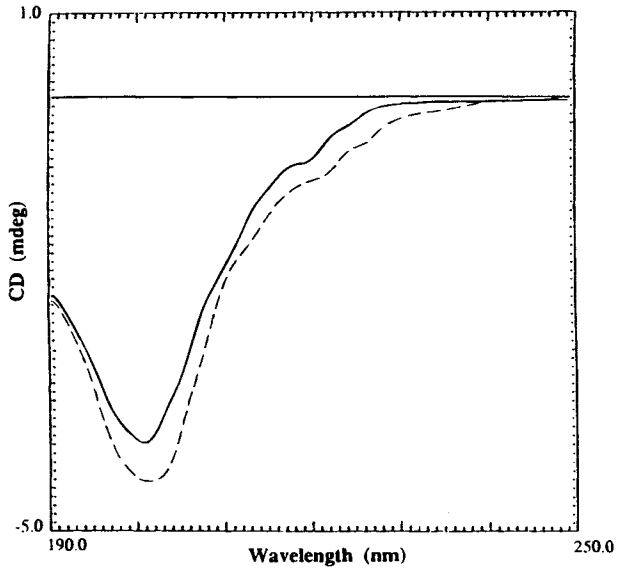

Fig. 4. CD spectra of hCT reference (dotted line) and hCT collected after loading in beads composed of the polymer having NiPAAm/BMA/AA mol ratio of 80/10/10 (full line). HCT concentration was 0.5 mg/ml.

Release

The release of hCT from the polymeric beads was investigated at different pH. The release was studied at pH 4.5 to avoid any aggregation of hCT during the experiment, and the release was also studied at pH 7.4 in order to simulate in vivo conditions. Samples from the release medium were collected at preselected time intervals and analyzed by HPLC. The results for hCT release from hydrophobic polymer (90/10/0) and hydrophilic polymer (80/10/10) in 10 mM acetate buffer pH 4.5, 0.15 M NaCl at 15°C are shown in Table I and Figure 6. The temperature of 15°C was selected to stay close, nevertheless below the LCST (18°C—see Fig. 1) of the polymers at pH 4.5 to allow for very slow dissolution of beads. Almost all the loaded hCT was released from the hydrophilic polymer and the time course of the hCT release correlated with a progressive swelling of the beads (up to 240 minutes), as determined by the diameter change in time (Fig. 6). In the case of the hydrophobic

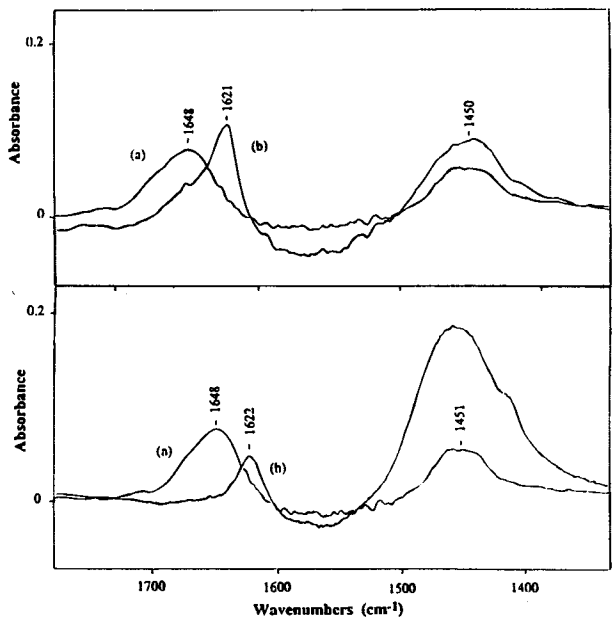

Fig. 5. Analysis of hCT structure by FTIR. Upper part: FTIR spectra of hCT reference (a) and hCT after 24 h in 20 mM glycine/DCl buffer, 0.15 M NaCl in D_2O (b). Bottom part: FTIR spectra of hCT reference (a) and hCT collected after loading in beads (NiPAAm/BMA/AA mol ratio = 80/10/10) (b).

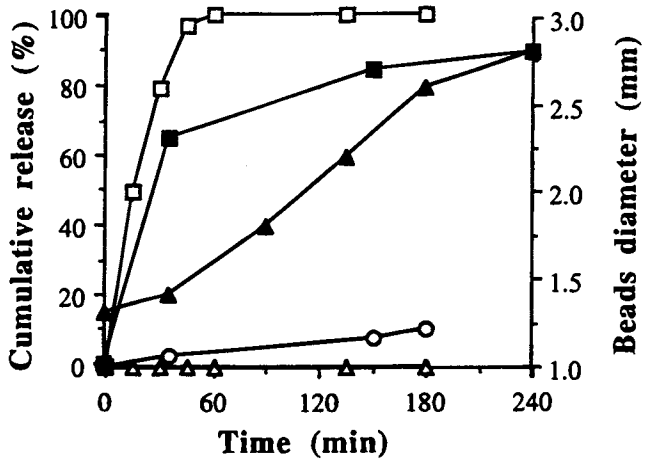

Fig. 6. Release of hCT from hydrophilic and hydrophobic beads at two different pH's. The percent of cumulative release is based on the amount of loaded hCT. The amount of released hCT from hyrophilic beads (NiPAAm/BMA/AA mol ratio = 80/10/10) at pH 4.5 and 15°C (■) correlates with the swelling of the beads (▲) while no correlation for hCT release from hyrophobic beads (NiPAAm/BMA/AA mol ratio = 90/10/0) (○) was observed. The change of diameter of hydrophobic beads is not shown for the reasons explained in the text. Release of hCT from hydrophilic beads (□) at pH 7.4 and 37°C was quantitative while no release from hydrophobic beads (△) under these conditions was detected. See text for further details.

polymer, a similar increase of beads diameter occurred (not shown) but dissolution rate was higher since beads started to disintegrate at 180 min. In spite of this, the release quantity of hCT from hydrophobic beads was very small indicating that hCT was already aggregated inside the beads.

Hydrophilic polymers evidently demonstrated better loading efficiency and allowed for the release of the major part of loaded hCT at pH 4.5. As a second step, the release experiments with the same polymers were performed in an isotonic phosphate buffer, pH 7.4 at 37°C (Fig. 6) to simulate physiological conditions. Practically all the loaded calcitonin was released within 60 min for the hydrophilic polymer. The beads dissolved at the same rate. In the case of the hydrophobic polymer with no acrylic acid, no release occurred under these conditions. Parallelly, no degradation of the hydrophobic beads was observed at pH 7.4 over several days in accord with corresponding LCST (13°C).

Table I. Loading Efficiencies of Polymer Beads and Release of hCT from Beads at pH 4.5 and 15°C

Feed composition NiPAAm/BMA/AA (mol ratio)	Loading efficiency (%) by		% hCT released
	HPLC	AAA[c]	
90/10/0	15 ± 4	11	15 ± 7[a]
87/10/3	19 ± 3	nd	nd
83/10/7	50 ± 6	54	nd
80/10/10	49 ± 3	nd	85 ± 4[b]

[a] Cumulative amount of released hCT after 3 hours.
[b] Cumulative amount of released hCT after 4 hours.
[c] AAA—Amino acid analysis.
Loading efficiency was determined by HPLC and amino acids analysis. The determination of the percent of released hCT (by HPLC) is based on the amount of loaded hCT. Values obtained by HPLC are expressed as mean of three to five experiments ± S.D. nd: not determined.

Bioactivity

The bioactivity of hCT recovered from the beads after loading and release *in vitro* was tested in rats. To determine hCT bioactivity after loading, the beads were solubilized at pH 3.0 and polymer precipitated (see above) to obtain pure hCT.

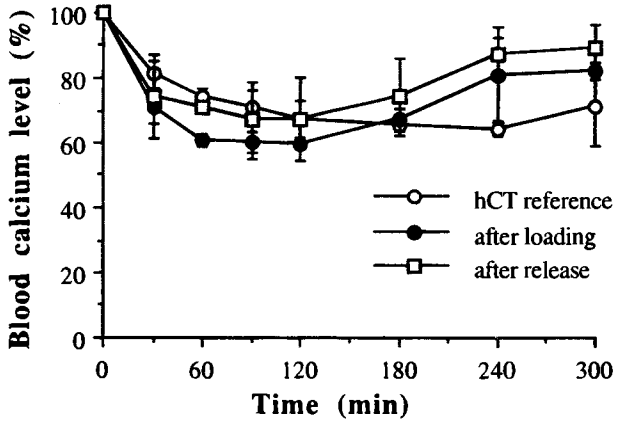

Fig. 7. Bioactivity of hCT recovered for the determination of loading efficiency and hCT released at pH 7.4 and 37°C (60 minutes) from the beads with NiPAAm/BMA/AA mol % ratio 80/10/10 and compared to bioactivity of hCT reference solution (see insert). Vertical bars represent standard deviation (n = 4). See Experimental section for further details.

The supernatant containing hCT was diluted and the pH was adjusted to neutrality. The appropriately diluted solution was injected intramuscularly into the rats. Blood samples were collected at different times and the plasma calcium levels were determined. Similar protocol was used to determine the bioactivity of released calcitonin. Plasma calcium levels versus time are shown in Fig. 7. In comparison with a hCT solution prepared just before injection, the bioactivity of recovered hCT after loading or release from polymeric beads was preserved.

CONCLUSIONS

pH/Temperature-sensitive polymers poly(NiPAAm-co-BMA-co-AA) were used to prepare human calcitonin releasing devices via loading in aqueous solution. The physical instability of the hormone required the study of loading conditions in the polymeric beads. It was shown that the hydrophilic polymer (high content of acrylic acid) provided high loading efficiency and release of human calcitonin. After prolonged contact with this polymer, human calcitonin was still bioactive in vivo (rat) and very likely nonaggregated. It may be that acrylic acid residues in the hydrophilic polymer provide for local acidic environment in the beads required generally for stabilization of hCT in both, solid state and in solution. It would be interesting to further study the interactions of this polypeptide and the copolymer in order to understand in more detail the ability of the hydrophilic polymer to stabilize this drug. According to the described results, these polymeric systems may be a way to improve calcitonin therapy by oral administration and to decrease side effects of the currently used administration routes. However, success of such oral systems depends on finding of suitable enhancers for calcitonin intestinal absorption (24).

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