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Polyisobutylcyanoacrylate nanoparticles as sustained release system for calcitonin

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Abstract

The potential of nanoparticles of polyisobutylcyanoacrylate as sustained release for peptide was assessed using calcitonin as a model drug. Calcitonin-loaded nanoparticles were obtained following the addition of the peptide before or after polymerization of isobutylcyanoacrylate (CT-NP and CT/NP, respectively). For both formulations, the percentage of binding of calcitonin to the nanoparticles was more than 95% and the particles had an average size of 150 nm. In vitro studies indicate that the release of calcitonin from CT/NP in saline solution containing esterases resulted from the bioerosion of the polymer. However the peptide was not released from CT-NP in this medium. SDS-PAGE electrophoresis and HPLC also showed that calcitonin is tightly bound to the nanoparticles in the CT-NP formulation, very likely by a covalent binding. After intravenous injection in rats, free calcitonin, CT/NP and CT-NP had the same hypocalcemic activity (at the same dose). Following a subcutaneous injection in rats, the two encapsulated forms of calcitonin showed a more important and more prolonged hypocalcemic effect than free calcitonin. 1 h after injection, calcitonin level was lower after CT-NP injection than CT/NP or free calcitonin injection. However, calcitonin level was sustained for more than 24 h after CT-NP injection.

Keywords: Calcitonin; Nanoparticle; Polyalkylcyanoacrylate; Sustained release

1. Introduction

Calcitonin is a polypeptide of 32 amino acids which has been shown to inhibit osteoclast activity. It is an effective therapeutic agent used in the management of several disorders characterized by an accelerated bone resorption. These include Paget's disease, postmenopausal osteoporosis and malignant hypercalcemia [1].

Calcitonin, as other peptides, is easily degraded by proteolytic enzymes in the gastrointestinal tract and therefore has to be administered by injections. The commercial preparation of human calcitonin, Cibacalcin®, is an aqueous solution of the peptide for intramuscular or subcutaneous injection. However, calcitonin has a short half-life in the body and the major drawback for the use of Cibacalcin® is the necessity of frequent parenteral administrations.

To avoid the inconvenience of injections, new galenic forms have recently been developped. It has been demonstrated that calcitonin is absorbed through the nasal mucosa. A nasal formulation of salmon calcitonin is now a marketed product in several countries [2–4]. Other routes of administration, such as rectal, ocular administration and transdermal delivery by iontophoresis are also considered as alternative modes of delivery [5–7].

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Sustained release systems have been investigated to avoid frequent administrations. Previous studies have already demonstrated the efficacy of liposomes and microspheres prepared with poly-(α-hydroxy acid) to prolong the hypocalcemic activity of calcitonin [8–10].

Polyalkylcyanoacrylate nanoparticles are of interest as possible carriers for the controlled release of drugs [11]. The aim of this study was to assess these nanoparticles as sustained release system for peptides using calcitonin as a model drug. After formulation of calcitonin loaded nanoparticles, physicochemical characterization as well as in vitro release and in vivo efficacy of calcitonin were studied.

2. Materials and methods

Cibacalcin® (a lyophilized product containing human calcitonin and mannitol) was obtained from Ciba (Groot Bijgaarden, Belgium) and [^{125}I]-calcitonin from Amersham (Aylesbury, UK). Dextran 40 was from Pharmacia (Uppsala, Sweden) and the Hank's Balanced Saline Solution from Gibco (Paisley, UK). Isobutylcyanoacrylate was purchased from Ethicon (Nordestedt, Germany). Esterases were from Prolabo (Paris, France). Glucose, glycerol, Tris-HCl, acetic acid, β-mercaptoethanol, citric acid and sodium dodecylsulfate were acquired from Merck (Darmstadt, Germany). Glycine and the solvents (methanol and acetonitrile HPLC grade) were from UCB (Braine-l'Alleud, Belgium). Acrylamide, *N'*,*N'*-bis-methylene-acrylamide, ammonium persulfate, bromophenol blue, Temed and Coomassie Brilliant blue were obtained from Bio-Rad (Richmond, USA). Wistar rats were purchased from Iffa Credo (St Germain-les-Arbresle, France).

2.1. Preparation of calcitonin-loaded nanoparticles

2.1.1. Polymerization process

Two different formulations of calcitonin-loaded nanoparticles were prepared. They were called CT-NP and CT/NP, depending on the moment of addition of calcitonin to the polymer, before or after polymerization, respectively. To avoid peptide degradation by bacterial proteases, preparation of calcitonin loaded nanoparticles were performed under sterile conditions [12].

A. Preparation of CT/NP. Isobutylcyanoacrylate monomer (120 μl) was added under mechanical stirring to 9 ml of an aqueous medium (dextran 40 1%, glucose 5%) adjusted to pH 2.8 with citric acid. After 4 h of polymerization, unloaded nanoparticles were obtained. Calcitonin was dissolved in the aqueous medium at a concentration of 1 mg/ml with 0.4 μCi/ml of ^{125}I-labelled calcitonin and 1 ml of this solution was added to the suspension of unloaded nanoparticles. This new suspension was maintained under mechanical stirring for 2 h.

B. Preparation of CT-NP. Isobutylcyanoacrylate monomer (120 μl) was added under mechanical stirring to 10 ml of the acidic medium mentioned above containing calcitonin at a concentration of 100 μg/ml with 0.04 μCi/ml of ^{125}I-labelled calcitonin. Magnetic stirring was maintained for 5 h.

Nanoparticles were freeze-dried for 48 h in a Lyovac GT 2 lyophilizator (Leybold-Heraeus, GmbH, Koln, Germany) and maintained at −20°C. The suspensions of nanoparticles were reconstituted just before use by addition of water for injection to the freeze-dried products.

2.1.2. Determination of the size of the nanoparticles

The mean value and the coefficient of variation of the nanoparticles size were determined before and after the freeze-drying by laser light-scattering measurement (Coulter N4, Coulter Electronics, Hialeh, Florida, USA).

2.1.3. Determination of the percentage of calcitonin bound to the nanoparticles

The suspension of nanoparticles was centrifugated at 100,000 × *g* for 90 min at 4°C and the radioactivity of the supernatant was measured by gamma counting (Autogamma Scintillation Spectrometer, Packard). The percentage of binding was determined as followed:

$$100 \times \left(1 - \frac{\text{radioactivity of the supernatant}}{\text{total radioactivity}}\right)$$

2.2. In vitro release of calcitonin from the nanoparticles

The calcitonin-loaded-nanoparticles (CT-NP and CT/NP) (25 μg of calcitonin, 0.1 μCi, 3 mg of poly-

mer) were suspended in 1 ml of Hank's balanced saline solution (HBSS) (pH 7.4) containing or not containing esterases (150 μg/ml). The release studies were performed in shaking tubes at 37°C. Periodically, samples of 1 ml were collected. 200 μl were removed to determine the total radioactivity. The remaining 800 μl were centrifugated at 100,000×*g* for 90 min at 4°C and the radioactivity of the supernatant was measured. The percentage of the drug released from the nanoparticles was estimated by calculating:

$$\frac{\text{radioactivity in the supernatant} \times 1.25}{\text{total radioactivity} \times 5} \times 100$$

2.3. In vitro degradation of the nanoparticles

The calcitonin-loaded nanoparticles (CT-NP and CT/NP) and unloaded nanoparticles were incubated in the same conditions as for the in vitro release. At different times, the turbidity of the suspensions was measured at 400 nm to follow the degradation of the nanoparticles.

2.4. Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed to compare the binding of calcitonin to the nanoparticles in the two formulations (CT-NP and CT/NP).

An acrylamide/*N'N'*-bis-methylene-acrylamide (19:1) mixture was dissolved in water at a concentration of 30% (p/v). The running gel was prepared after addition of a calculated amount of this mixture to a degassed Tris-HCl buffer (pH 8.8, 0.15 M) to obtain a gel concentration of 20% (p/v). Ammonium persulfate (0.1%) and Temed (0.05%) were used as initiator and catalyst of the polymerization, respectively. The stacking gel was prepared in a Tris-HCl buffer (pH 6.8) and at a concentration of 4% (p/v). The different samples (Cibacalcin®, CT-NP, CT/NP and unloaded nanoparticles) were suspended or dissolved in a sample buffer (Tris-HCl 0.0625 M, glycerol 10% (v/v), sodium dodecylsulfate 21% (p/v), β-mercaptoethanol 5% (v/v), bromophenol blue 0.00125% (p/v)) at a concentration of 10 mg of calcitonin/ml and 1.2 g of polymer/ml. 20 μl of the suspension of each sample were loaded into the wells of the gels. The running buffer was composed as follows, Tris-HCl 0.025 M, glycine 0.192 M and sodium dodecylsulfate 0.1%. Electrophoresis was performed under a constant voltage of 200 V (Mini Protean II®, Bio-Rad). Gels were then placed in a staining solution (Coomassie brilliant blue 0.25%, methanol 30% and acetic acid 7% in water) for 30 min–1 h. Afterwards, the gel was destained in an aqueous solution containing methanol (30%) and acetic acid (7%) [13].

2.5. HPLC method

The HPLC system consisted of two solvent delivery systems (model 6000A, Waters), a gradient controller (Waters), an injector (model WISP 710B), a microbondapak C18 column (3.9 mm×30 cm) (Waters) and a variable wavelength UV detector (model 491, Waters).

Human calcitonin and its degradation products were detected at 230 nm. A linear gradient was used: 25% A 75% B to 50% A 50% B over 25 min. Mobile phase A was a 0.1% trifluoroacetic acid/acetonitrile solution and mobile phase B was 0.1% trifluoroacetic acid/distilled water. The injection volume was 20 μl and the flow rate was 1 ml. Standard curves with 2.5 to 50 μg/ml calcitonin were performed [10,14]. Nanoparticles and calcitonin Cibacalcin® were diluted in acetonitrile/water 4:1 in which nanoparticles were soluble.

2.6. Animal experiments

Male Wistar rats weighing 200–260 g were housed in individual cages. The animals were maintained in conditions of 12 h-day light cycles and had free access to food and water. They were randomly assigned to treatment and control groups. The free calcitonin and the 2 formulations of calcitonin-loaded nanoparticles (CT-NP and CT/NP) were dissolved or suspended in water for injection just before use. The rats were injected intravenously or subcutaneously with the different formulations of calcitonin (10 μg/kg and 50 μg/kg body weight respectively). Blood samples were collected from the tail veins of the animals at different times after the administration of the drug and the sera were frozen until analysis for their calcium or calcitonin content.

Calcium serum concentrations were then determined by atomic absorption (Perkin-Elmer 375).

Serum calcitonin were measured by R.I.A. using a polyclonal anti human calcitonin antiserum kindly provided by J.A. Fischer (University of Zurich, Switzerland) and synthetic human calcitonin (Peninsula laboratories, UK) as standard [15]. The sensitivity of the method is 50 pg/ml.

3. Results

3.1. Characterization of the calcitonin-loaded nanoparticles

The size of the CT-NP and CT/NP nanoparticles was 149 nm ± 20% and 121 nm ± 20%, respectively.

Table 1
Percentage of binding of calcitonin to PIBCA nanoparticles and mean size of the particles, before and after freeze-drying

Formulations ($n=8$)	Size of the nanoparticles (mean (nm) ± C.V.%)	Percentage of binding of calcitonin to the nanoparticles
CT-NP		
Before freeze-drying	149 ± 20%	97.9 ± 0.8
After freeze-drying	164 ± 20%	97.7 ± 1.5
CT/NP		
Before freeze-drying	121 ± 20%	96.3 ± 1.0
After freeze-drying	139 ± 27%	95.9 ± 1.2

CT-NP, calcitonin added before the polymerization of the nanoparticles. CT/NP, calcitonin added after the polymerization of the nanoparticles. The concentrations of calcitonin and of the polymer were 100 μg/ml and 12 mg/ml, respectively (for details, see Material and methods).

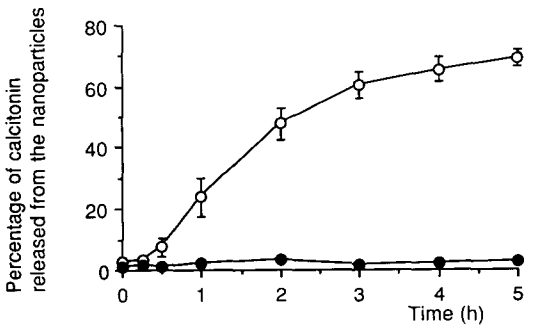

Fig. 1. Percentage of calcitonin released from CT-NP (close circle) or CT/NP (open circle) in Hank's balanced saline solution (pH 7.4) containing esterases (150 μg/ml). The concentrations of calcitonin and of polymer in the medium were 25 μg/ml and 3 mg/ml, respectively. Data are presented as means ± SEM ($n=3$).

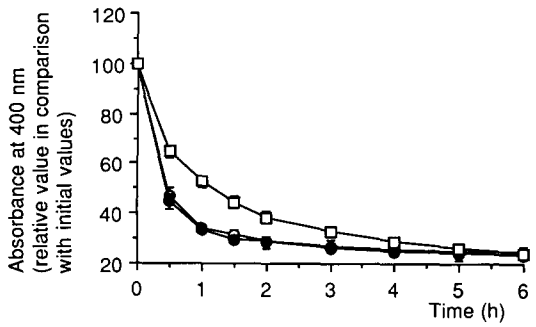

Fig. 2. Evolution of the turbidity of unloaded nanoparticles (open circle), CT/NP (close circle) or CT-NP (open square) at 400 nm in Hank's balanced saline solution (pH 7.4) containing esterases (150 μg/ml). The concentrations of calcitonin and of polymer in the medium were 25 μg/ml and 3 mg/ml, respectively. Data are presented as means ± SEM ($n=3$).

The percentage of binding of calcitonin to the nanoparticles was calculated as 97.9% for the CT-NP formulation and 96.3% for the CT/NP formulation. Freeze-drying did not modify the size and the percentage of binding of these calcitonin-loaded nanoparticles (Table 1).

3.2. In vitro release from nanoparticles and nanoparticles degradation

When incubated in HBSS, no calcitonin was released from the two formulations of nanoparticles (CT-NP and CT/NP) after 5 h (data not shown).

In the presence of esterases, calcitonin was released progressively from CT/NP during the 5 h of incubation (Fig. 1). Simultaneously, the turbidity of the suspension progressively decreased. Measurements of turbidity also showed that the degradation of these nanoparticles was similar to unloaded nanoparticles degradation (Fig. 2).

In contrast, calcitonin was not released from CT-NP after 5 h of incubation and the turbidity of this nanoparticular suspension decreased less rapidly than the turbidity of unloaded nanoparticles or CT/NP suspensions. These results suggest that calcitonin is strongly linked to the polyisobutylcyanoacrylate polymer, very likely by a covalent binding.

3.3. Electrophoresis

The aims of this experiment were to compare the molecular weight of calcitonin from the two formula-

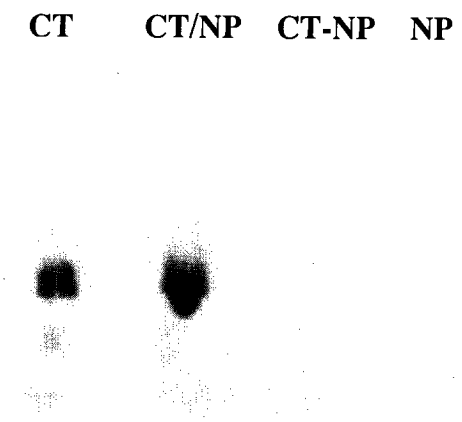

Fig. 3. SDS-polyacrylamide gel electrophoresis of different formulations of calcitonin (for details see Material and methods); Lane 1, free calcitonin CT; Lane 2: CT/NP; Lane 3, CT-NP; Lane 4, unloaded nanoparticles NP.

tions (CT-NP and CT/NP) relative to free calcitonin and to evaluate the binding of calcitonin to the nanoparticles.

As shown in Fig. 3, calcitonin in the CT/NP formulation was released from the nanoparticles and migrated in the gel as free calcitonin did. In contrast, no band was detected for the CT-NP, suggesting a strong binding of calcitonin to the polyisobutylcyanoacrylate polymer which did not migrate in the gel.

3.4. HPLC studies

In order to check if calcitonin was not degraded during nanoparticles preparation and freeze drying, a reverse phase gradient HPLC was performed [10,14].

When CT/NP nanoparticles were analyzed before and after freeze drying, the amount of calcitonin measured corresponded to expected values. Indeed, the peak areas of calcitonin from CT/NT or free calcitonin at the same concentration was equal. No degradation product of calcitonin was detected. HPLC chromatograms of CT-NP did not show any calcitonin nor a degradation product, suggesting a strong binding associated or not with a degradation of calcitonin.

3.5. In vivo studies

The hypocalcemic effects in rats of free calcitonin, CT-NP and CT/NP, are illustrated in Figs. 4, 5 and Table 2.

The intravenous injection of 10 μg calcitonin/kg induced a 20% decrease in the calcium serum concentration after 2 h. Afterwards, calcemia increased rapidly to control values 5 h after the injection.

The intravenous injection of calcitonin encapsulated into nanoparticles (CT-NP or CT/NP, 10 μg calcitonin/kg and 1.2 mg polymer/kg) induced a similar profile in the serum calcium concentrations as the intravenous injection of free calcitonin.

Following a subcutaneous injection of free calcitonin (50 μg/kg), a 12% decrease in the calcemia of the

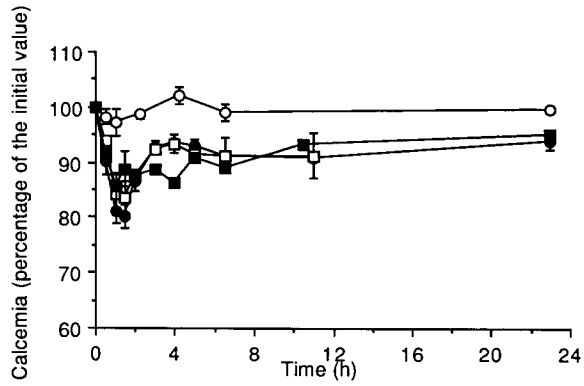

Fig. 4. Evolution of the calcemia of the rats following the intravenous administration of different formulations of calcitonin (10 μg calcitonin and/or 1.2 mg polymer/kg). Controls received water for injection. Controls (open circle); free calcitonin (closed circle); CT/NP (open square); CT-NP (closed square). Data are presented as means ± SEM ($n=5$).

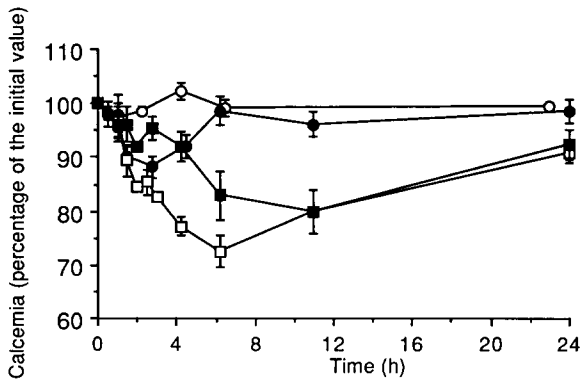

Fig. 5. Evolution of the calcemia of the rats following the subcutaneous injection of different formulations of calcitonin (50 μg calcitonin and/or 6 mg polymer/kg). Controls (open circle); free calcitonin (closed circle); CT/NP (open square); CT-NP (closed square). Data are presented as means ± SEM ($n=5$).

Table 2
Minimum calcemia and time to obtain this minimum calcemia in rats following an intravenous of a subcutaneous administration of different formulations of calcitonin in rats (10 μg calcitonin/kg and 50 μg calcitonin/kg, respectively)

Formulations	Time after injection (h)	Minimum calcemia (mean ± SEM; $n=5$) (Percentage of the initial value)	Mode of administration
Free calcitonin	1.5	80.2 ± 2.1	intravenous
CT/NP	1.5	83.6 ± 2.9	intravenous
CT-NP	1	85.7 ± 2.5	intravenous
Free calcitonin	2.75	88.1 ± 2.0	subcutaneous
CT/NP	6.25	72.5 ± 2.9	subcutaneous
CT-NP	11	80 ± 0.9	subcutaneous

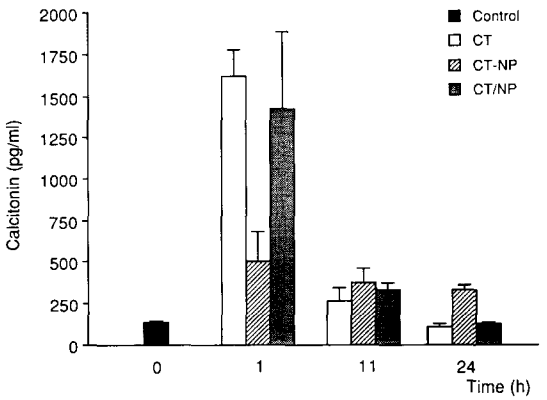

Fig. 6. Calcitonin concentration 1 h, 11 h and 24 h after the subcutaneous injection of free calcitonin (50 μg/kg), CT/NP or CT-NP (50 μg/kg calcitonin, 6 mg PIBCA/kg). Data are presented as mean ± SEM ($n=4$).

animals was observed 2.75 h after the administration of the drug. Thereafter, the calcemia increased rapidly to the initial value within 6 h after injection. The calcitonin loaded nanoparticles induced a more important and more prolonged hypocalcemic effect. After the subcutaneous administration of CT/NP, a 28% decrease in the animals calcemia was observed 6.25 h after the administration of the drug. Then calcemia progressively increased and reached 90% of the initial value after 24 h. The subcutaneous injection of CT-NP also decreased the calcemia of the animals. However, in comparison with CT/NP, the maximal effect is observed later and is less important (a 20% decrease after 11 h).

Extended hypocalcemic effects are suitable only to see the hemodynamic effects and not pharmacological effects. The resurge of calcium levels does not indicate disappearance or elimination of calcitonin. Therefore, calcitonin concentrations were measured 1 h, 11 h and 24 h after free or encapsulated calcitonin injection.

1 h after injection, calcitonin level was lower after CT-NP injection than CT/NP or free calcitonin administration. However, increase in calcitonin levels was sustained as compared to control value for more than 24 h after CT-NP injection (Fig. 6).

These results indicate that the delivery of calcitonin is more sustained from CT-NP where calcitonin is tightly bound to PIBCA than from CT/NP.

4. Discussion

Calcitonin has a high affinity for polyisobutylcyanoacrylate nanoparticles and this study shows that whatever the moment of addition of the peptide, before or after polymerization, or the pH of the medium, pH 2.8 or pH 6.5 (data not shown), a high percentage of drug binding was obtained. In this study we showed that nanoparticles with efficient loading of calcitonin and suitable size for i.v. injection were easily prepared under sterile conditions.

Bioerosion and not passive diffusion was responsible for the release of calcitonin from the CT/NP nanoparticles: without esterases in the medium, no release of calcitonin occurred. In the presence of esterases which are responsible for the bioerosion of polyalkylcyanoacrylate by hydrolysing the ester link of the side chain [16], the turbidity of the nanoparticular suspension decreased and the drug was released.

Calcitonin is a peptide and contains amino groups in its structure. Previously, it had been shown that molecules with an amino group in their structure could form a covalent binding with polyalkylcyanoacrylate nanoparticles when added to the medium before polymerization [17,18]. Different results obtained in this study suggest that such a binding exists between calcitonin and polyisobutylcyanoacrylate nanoparticles in the CT-NP formulation but not in the CT/NP formulation:

- even in the presence of esterases, calcitonin was not released from the CT-NP nanoparticles (Fig. 1).
- the degradation of the CT-NP nanoparticles was modified in comparison with that of unloaded nanoparticles and CT/NP nanoparticles (Fig. 2).

- SDS-polyacrylamide gel electrophoresis experiment showed that the calcitonin of this CT-NP formulation did not migrate in the gel (Fig. 3).
- no calcitonin was detected in HPLC chromatogram of CT-NP nanoparticles.

Grangier et al. [17] recently showed that GRF (Growth hormone releasing factor) can be associated to polyalkylcyanoacrylate nanoparticles. The moment at which GRF was incorporated in the polymerization medium was a critical factor. When added at the beginning of polymerization, GRF was also covalently bound to the polymer. However, the biological activity of this formulation of GRF-loaded nanoparticles was not measured to check if GRF activity was maintained after such covalent binding. In our studies, calcitonin retained its hypocalcemic effect despite its strong binding to the polymer and plasma levels were increased for more than 24 h.

In this study, the in vivo efficiency of calcitonin loaded PIBCA nanoparticles was assessed by measuring the hypocalcemic effect. Following an intravenous injection in rats of 10 μg/kg, free calcitonin, CT/NP and CT-NP showed the same hypocalcemic activity. Similar results were previously obtained with nanoparticles containing GRH: the GRH blood concentrations after i.v. administration of free or encapsulated GRH were equivalent. This could be attributed to the rapid capture of the nanoparticles by the reticuloendothelial system [19].

After a subcutaneous administration, PIBCA nanoparticles can be used as a reservoir which acts as a sustained released system for calcitonin: the subcutaneous injection of CT/NP and of CT-NP induced in rats more prolonged and more important hypocalcemic activities than the administration of free calcitonin. Moreover, calcitonin delivery was sustained after encapsulation of the peptide for more than 24 h after CT-NP injection. In addition to the slow release process, nanoparticles were able to improve the bioavailability of the peptide. This could be explained by the fact that nanoparticles protect the peptide against enzymatic degradation [19]. The delayed activity observed with CT-NP and prolonged delivery of calcitonin in comparison with CT/NP could be attributed to the tighter binding of calcitonin to polyisobutylcyanoacrylate nanoparticles (Figs. 4, 5, 6, Table 1).

In conclusion, the results demonstrate the potential of nanoparticles of PIBCA as a sustained release system for calcitonin delivery after subcutaneous administration.

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