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Novel vaginal delivery systems for calcitonin II. Preparation and characterization of HYAFF® microspheres containing calcitonin

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Abstract

Microspheres prepared from hyaluronan esters (HYAFF®) have been evaluated as a novel delivery system for the vaginal administration of salmon calcitonin (sCT). HYAFF® microspheres containing sCT were prepared by a solvent extraction method. Spherical microspheres of about 10 mm in diameter with smooth surfaces were obtained. An HPLC method was developed for the determination of sCT incorporated into the microspheres. The efficiency of incorporation was high, with approximately 80 to 90% of the peptide recovered by extraction from the microspheres. Quantification of the extracted peptide in vivo confirmed that the biological activity of sCT was unaffected by the microsphere preparation process.

Keywords: Vaginal administration; Calcitonin; Hyaluronan; Microspheres; Drug delivery systems

1. Introduction

Calcitonin (CT) is a polypeptide hormone consisting of 32 amino acids arranged in a single chain and is characterised by a disulphide bridge between the cysteine residues at positions 1 and 7 and by a carboxy-terminal prolinamide. CT is known to inhibit bone resorption and to play a role in calcium homeostasis and, consequently, is used in the treatment of postmenopausal osteoporosis, Paget's disease and hypercalcaemia (Deftos, 1978). Among calcitonins available for clinical use — salmon (sCT), human, eel and porcine calcitonin — sCT is the most potent (Guttman, 1980) and is the most widely used (Carstens and Feinblatt, 1991).

Due to its low bioavailability after oral administration, sCT is generally administered by subcu-

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taneous or intramuscular injection. However, the disadvantages of injectable formulations of sCT include a high incidence of systemic side effects, inconvenience of repeated injections and a resulting non compliance. Currently, two avenues of research are being pursued to improve calcitonin therapy. The first is focused upon the development of sustained release delivery systems for injection or implantation and the second concerns the exploitation of mucosal routes of administration. Following the former approach, polymeric matrices such as poly(glycolic acid) microspheres (Lee et al., 1991a) and poly(DL-lactic acid) cylinders (Asano et al., 1993) have been studied as sustained release delivery systems for sCT and eel CT, respectively. Alternatively, utilising more simple formulations, the potential of nasal and rectal (Buclin et al., 1987), colonic (Beglinger et al., 1993) and vaginal (Nakada et al., 1993) routes of administration for CT has been assessed.

Generally, peptides and proteins display low bioavailabilities from mucosal sites due to their poor absorption and susceptibility to enzymatic degradation. Various ways of improving peptide and protein delivery have been studied, including use of absorption enhancers, bioadhesive delivery systems and enzyme inhibitors (for review see O'Hagan and Illum, 1990). Our approach has been the development of microspheres composed of hyaluronan esters (HYAFF®) for the vaginal administration of sCT. Acute vaginal administration of the HYAFF®/sCT microspheres resulted in pronounced hypocalcaemia in rats (Richardson et al., 1995) and daily administration for two months was shown to prevent bone loss in a rat model for osteoporosis (Bonucci et al., 1995). HYAFF® microspheres have previously been evaluated as delivery devices for nerve growth factors (Ghezzo et al., 1992) and as a nasal delivery system for insulin (Illum et al., 1994).

The present paper describes the preparation of HYAFF® microspheres containing sCT. Hyaluronan (HA) is a naturally occurring mucopolysaccharide consisting of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine. By esterification of the carboxyl groups of the glucuronic acid residue with alcohols, it has been possible to produce modified biopolymers with physical properties that are significantly different from those of HA itself (Benedetti et al., 1991). These esters are biocompatible (Campoccia et al., 1993), mucoadhesive and biodegradable: the degradation rate being dependent on the degree of esterification and on the alcohol substituent used. HYAFF® 11 is prepared by the total esterification of HA with benzyl alcohol, rendering the polymer insoluble in water and in many common organic solvents. HYAFF® 11 is however soluble in some aprotic solvents such as dimethylsulphoxide (DMSO). Consequently, these semi-synthetic derivatives of HA may be developed into a versatile range of biomaterials and delivery devices by extrusion or extraction methods.

The preparation of HYAFF® microspheres containing sCT is described below and in particular the development of analytical methods for the extraction and determination of the sCT content of the microspheres is presented. In addition, the effect of the microsphere preparation method on the integrity of the peptide was assessed by quantification of its pharmacological activity in vivo by measurement of hypocalcaemia after intravenous administration to rats.

2. Materials and methods

2.1. Materials

HYAFF® 11 (HA with all carboxyl group esterified with benzyl alcohol) with a molecular weight of approximately 180 kDa, was supplied by F.A.B. s.r.l. (Brindisi, Italy). Salmon calcitonin, with a biological activity 5772 IU/mg, was supplied by Bachem Inc (Torrance, USA). Mineral oil (Kaydol) was purchased from Sold_ (Vicenza, Italy). Arlacel® A (mannide monooleate) was obtained from Sigma Chemical Company (St. Louis, MO). Ethyl acetate, dimethylsulphoxide, *n*-hexane and acetonitrile were purchased from Rudi Pont (Eurobase S.p.A., S. Giuliano Milanese, Italy). Guanidine hydrochloride was purchased from Fluka Chemie (Buchs, Switzerland); monobasic sodium phosphate was obtained from Merck (Darmstadt, Germany). All reagents were analytical grade and were used without further

purification. The water was deionized using a Milli-Q system (Millipore). All solutions and buffers were prepared with deionized water that was degassed and filtered through a 0.45 mm membrane (Millipore) prior to use.

2.2. Preparation of microspheres

HYAFF® 11 microspheres containing sCT were prepared by a solvent extraction method (Benedetti et al., 1990) as follows: HYAFF® 11 was dissolved in DMSO at a concentration of 6% w/v. A solution of 2% w/v sCT in water was added to the polymer solution and the mixture was stirred for 20 min at 1000 rpm. using a mechanical stirrer (RW20, IKA WERK Instruments). The polymer/sCT mixture was added to mineral oil containing 0.5% w/v Arlacel A at a ratio of 1:16 v/v. An emulsion was formed by stirring with a disperser (KINEMATICA) at a rate of 10 000 rpm for 10 min.

Ethyl acetate, at a ratio of 2:1 v/v, was quickly added to extract the DMSO and mineral oil and to precipitate microspheres of HYAFF®/sCT. The suspended microspheres were filtered under pressure (1.5 atm) through a polyamide membrane using a steel filter fitted with a magnetic stirring shaft. The powder was resuspended twice in ethyl acetate and then twice in *n*-hexane to remove the excess mineral oil and surfactant. The microspheres were then dried under vacuum for 24 h.

2.3. Physical characterization

The shape and surface characteristics of microspheres were examined using a scanning electron microscope (Philips 505). Samples were prepared by sputter coating with gold in an Edwards Sputter Coater 5150 B. Micrographs were obtained at a magnification of 2000–4000 ×.

The particle size of the microspheres was measured using a light scattering technique. A small quantity of microspheres was suspended in ethyl acetate and analyzed using a Master Sizer MS 20 (Malvern).

2.4. Chemical characterization

The microspheres were analyzed to determine the percentage of esterification and the molecular weight of HYAFF® 11, the concentration of residual solvents (DMSO, *n*-hexane, ethyl acetate and mineral oil) and the sCT content.

2.4.1. Percentage of esterification

In order to determine the degree of esterification of HYAFF® 11, the microspheres were hydrolysed in a solution of 0.1 N NaOH at 60°C for 30 min, to cleave the ester bond between HA and the alcohol. The benzyl alcohol quantity was then determined by reversed phase high performance liquid chromatography (HPLC) using a stainless steel column ODS (PhaseSep, 5 cm × 4.6 cm, 3 mm). The mobile phase consisted of 82:18 v/v acetonitrile:water at a flow rate of 1 ml/min. Under these conditions, the retention time of benzyl alcohol was 4 min. The HPLC system consisted of a Perkin Elmer pump (mod. 410), an automatic sample injector (Gilson mod. 231) and a diode array UV detector (Perkin Elmer mod. 235) set at a wavelength of 254 nm and connected to a data acquisition system (Maxima 820, Waters).

2.4.2. Molecular weight determination

The molecular weight of HYAFF® 11 was determined by HPLC after solubilization of the microspheres in a solution of 0.1 M tetrabutylammonium bromide in DMSO. The HPLC system used consisted of a HPLC pump (Gilson mod. 307) equipped with a refractive index detector (mod Erma Erc-7515 A) and an autosampler injector (Gilson mod. 401). The data was analyzed using a Maxima 820 data acquisition and processing system. To separate the polymer, two columns of PLF gel Mixed-A (600 mm × 7.5 mm; 20 mm) were used. The mobile phase consisted of 0.1 M tetrabutylammonium bromide in DMSO. The molecular weight of the polymer was calculated using standards of HYAFF® with known molecular weights and molecular weight distributions.

2.4.3. DMSO and n-hexane determination

DMSO and n-hexane were determined under the same conditions by gas chromatography (G.C.). Microspheres were suspended in ethanol and after filtration, the solution was injected directly into the G.C. system. A Perkin Elmer 8700 gas chromatograph equipped with a flame ionization detector (FID) was used. Chromatographic separation was obtained using a stainless steel column (6 ft × $\frac{1}{8}$″, Altech) packed with 0.2% silar 10 C on graphpack G.C. (80–100 mesh). The following chromatographic conditions were used:

- oven temperature: 130°C
- injector temperature: 250°C
- FID temperature: 300°C
- carrier N_2: 15 psi
- volume of injection: 1 μl

Under these conditions the retention times of DMSO and *n*-hexane were 5 and 6 min, respectively.

2.4.4. Ethyl acetate determination

Microspheres were suspended in 1 ml of distilled water in a vial sealed with a rubber septum and aluminium cap. Ethyl acetate was determined using a gas chromatographic method with a head space technique. A Perkin Elmer 8700 G.C. equipped with a Perkin Elmer Head Space HS 101 and a FID was used. The chromatographic separation was performed using a stainless steel column (6ft × $\frac{1}{8}$″) packed with carbopack C/0.1% SP-1000 Supelco. The following conditions were used:

- oven temperature: 50–110°C at 10°C/min.
- injector temperature: 180°C
- transfer line: 90°C
- thermostatation temperature: 60°C for 60 min.
- FID temperature: 300°C
- carrier N_2: 15 psi

The retention time was 12 min under these conditions.

2.4.5. Mineral oil determination

Microspheres were hydrolysed with a 2 M hydroalcoholic KOH solution, at 100°C for one hour. The resultant solution was extracted several times with petroleum ether (40:60 v/v) and after total evaporation of the extracting solvent, the dry residue was weighed.

2.4.6. sCT loading of microspheres

Three methods were evaluated for the determination of sCT content into HYAFF® microspheres:

(1) extraction of the drug from the microspheres using a 0.9% NaCl solution, acidified to pH 1 with 1 M HCl;
(2) complete dissolution of the microspheres in DMSO;
(3) suspension of the microspheres in a 6 M solution of guanidine hydrochloride (GuHCl).

A sample of sCT microspheres, sufficient to provide a concentration of sCT between 10 and 35 mg/ml, was accurately weighed into a polypropylene vial. Depending on the method used (1, 2 or 3), 1 ml of acidic saline solution, DMSO, or GuHCl solution was added and the mixture was shaken vigorously (Supermixer, Continental Instrument). The sample was then mixed at 37°C for 30 min, at 1200 rpm (Thermomixer 5437, Eppendorf). Samples prepared using methods 1 and 3 were filtered through a 0.2 μm polysulphone membrane. The sCT content of the resulting solution obtained by the three methods was determined by HPLC as described below. A standard curve was constructed for each method over a range of 1 to 35 μg/ml using sCT treated under the same conditions as the microsphere samples.

2.4.7. sCT determination

The sCT was assayed by reversed-phase gradient HPLC. The system used comprised two pumps (Waters, model 510); an automatic sample injector (WISP 712, Waters). A tunable UV detector (Waters, model 484) was connected to a data acquisition system (Maxima 820, Waters). Chromatographic separations were carried out using a 15 cm × 4.6 mm delta-pak C18 column (Waters 300 Å, 5 μm). sCT was eluted from the column with a mobile phase consisting of:

(1) 25 mM phosphate buffer:acetonitrile (75:25), pH 2.5;
(2) 25 mM phosphate buffer:acetonitrile (60:40), pH 2.5.

The linear gradient profile was from 20 to 55% B in 10 min and the flow rate was 1 ml/min. The sCT in the eluate was monitored spectrophotometrically at a wavelength of 216 nm.

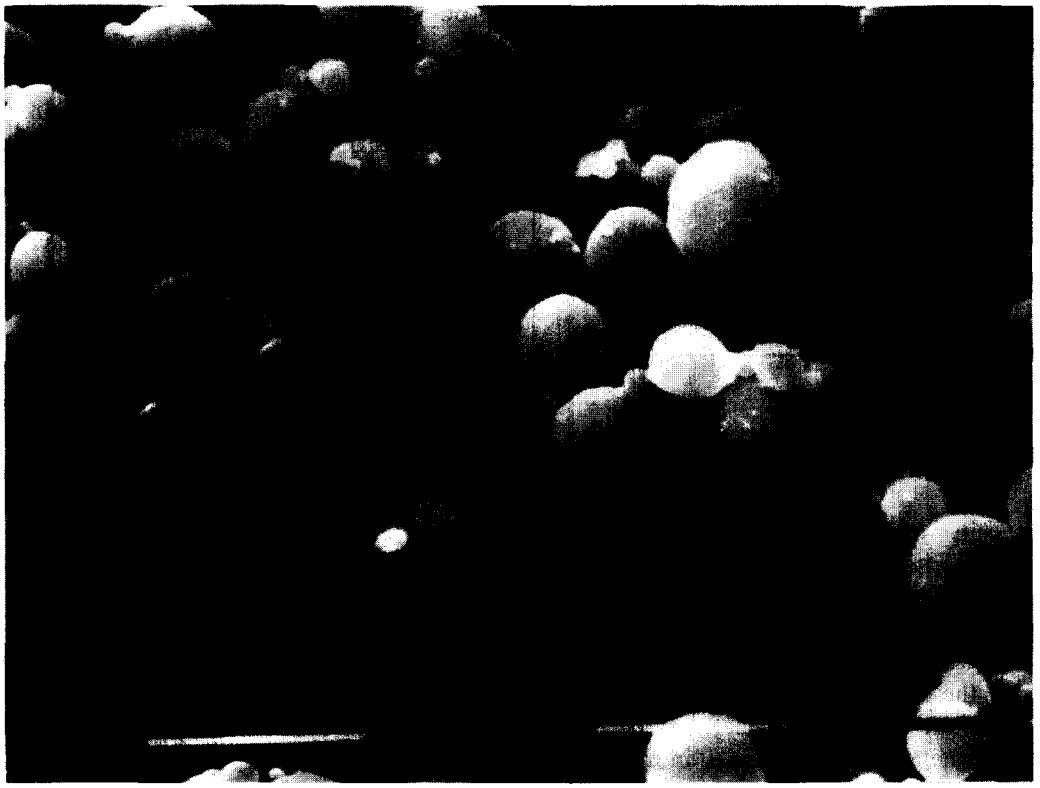

Fig. 1. Scanning electron micrograph of HYAFF® 11 microspheres loaded with salmon calcitonin. White bar represents 10 μm.

2.4.8. Determination of biological activity of sCT

To determine the biological activity of sCT, the peptide was extracted from the microspheres using the acidic saline solution or DMSO, as described above. The concentration of sCT in each sample was determined by HPLC.

The biological activity of sCT was assessed by measuring the decrease in plasma calcium levels after intravenous (i.v.) administration of sCT solution to rats (Assay of Biological Activity of Salmon Calcitonin, British Pharmacopoeia 1980). The two test samples of sCT in saline and DMSO were diluted with acetate buffer, pH 4.6, to 50, 25 and 12.5 mIU/ml. Two standard sCT solutions (National Institute for Biological Standards and Control, UK) were diluted similarly in DMSO and acetate buffer. Four groups of male Sprague Dawley rats ($n = 3$) weighing 60–80 g (Charles River Italia SPA, Calco) were used. The standard sCT solutions and test samples were injected intravenously via the tail vein at doses of 200, 100 and 50 mIU/kg (0.4 ml/kg). One hour after administration, blood samples were taken by cardiac puncture under anaesthesia. Blood samples were collected in heparinised tubes and plasma was separated by centrifugation. Plasma calcium levels were determined by atomic absorption spectrometry. The biological activity of the sCT in the two test samples was estimated by comparison of decreases in plasma calcium levels in animals treated with test samples and treated with the corresponding sCT standards.

3. Results and discussion

The HYAFF® 11 microspheres containing sCT were spherical in shape and had smooth surfaces (Fig. 1). The mean diameter of the microspheres was 7 mm (S.D. 0.5) and the particle size distribution tended to be narrow, as shown in Fig. 2.

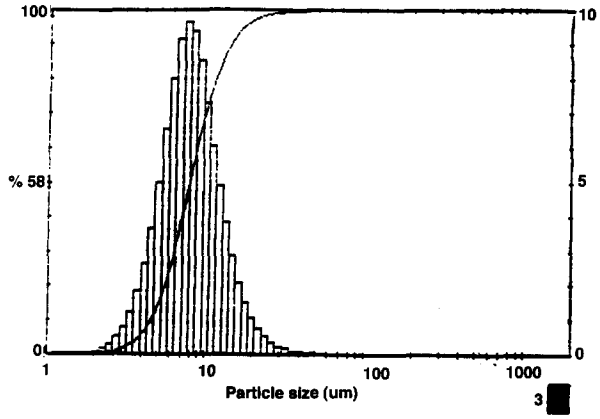

Fig. 2. Particle size distribution of HYAFF® 11 microspheres loaded with sCT.

The viscosity of the polymer solution was found to be a critical parameter that influenced the dispersion and the shape of the polymer droplets in the surrounding oil phase. Consequently, changes in polymer concentration caused variations in the size and physical characteristics of the microspheres. It was also necessary to limit the volume of the aqueous sCT solution mixed in the polymer/DMSO phase as when the ratio of water to DMSO exceeded 5% (v/v), a partial precipitation of the polymer occurred.

Other process parameters, such as rotor speed of the disperser and the time required for the addition of ethyl acetate to the emulsion influenced the aggregation of the polymer droplets and the formation of microspheres. Indeed, for the production of large batches of microspheres, a high rotor speed was employed to obtain HYAFF® 11/sCT microspheres within the desired size range. Furthermore, the addition of ethyl acetate to the polymer-mineral oil emulsion was performed as quickly as possible to avoid polymer aggregation.

The results of the chemical characterisation of three batches of HYAFF® 11/sCT microspheres are summarised in Table 1 (the differences in the degree of esterification and sCT concentration were related to a manufacturing difference). The molecular weight and degree of esterification of HYAFF® 11 was unaffected by the microsphere preparation process. The concentrations of residual solvents were less than 1% w/w for all the batches tested. Mineral oil was easily removed by washing with n-hexane; ethyl acetate and *n*-hexane were readily evaporated by vacuum drying and DMSO, which has a very high boiling point, was removed by repeated washing with ethyl acetate.

The concentration of sCT entrapped in HYAFF® 11 microspheres was determined by gradient HPLC using a method adapted from that of Lee et al. (1991b). The chromatographic conditions used, such as the concentration of acetonitrile, the ionic strength and the acidity of the mobile phase, were optimised to vary the retention time of sCT and enable separation of the peptide from the HYAFF® polymeric matrix, where necessary.

Table 1
Chemical characterization of three batches of HYAFF® 11/sCT microspheres

Test	Batch no. 1	Batch no. 2	Batch no. 3
Particle size (μm)	6.2	8.5	7.8
Loss on drying (%)	6.23	7.1	3.76
Polymer molecular weight (kD)	178	185	180
Polymer m.w. distribution (M_w/M_n)	1.9	2	2
Percentage of esterification (%)	93.0	95.2	85.2
Concentration of residual solvents:			
Ethyl Acetate (% w/w)	0.13	0.05	0.11
DMSO (% w/w)	0.4	0.7	0.9
Hexane (% w/w)	not detected	not detected	not detected
Acetone (% w/w)	not detected	not detected	not detected
Mineral oil (% w/w)	not detected	not detected	not detected

Table 2
sCT content of three batches of microspheres, as determined by three extraction methods. The concentrations of sCT recovered are expressed as μg/mg of microspheres and as a percentage of the initial quantity of peptide

Batch number	Theoretical concentration	Amount in acidic solution		Amount in DMSO solution		Amount in Gu–HCl solution	
	μg/mg	μg/mg	%	μg/mg	%	μg/mg	%
1	1.91	1.05	55.4	1.18	61.9	1.53	80.5
2	1.91	0.99	51.8	7.33	66.6	1.53	80.1
3	0.40	0.21	53.4	0.25	63.7	0.3	74.8

Three methods were studied for the extraction and measurement of sCT incorporated in HYAFF® 11 microspheres. At the some time the biological activity of sCT extracted from the microspheres was assessed in rats and compared with HPLC analysis of the same sCT samples. Since sCT is characterized by an increased solubility in acidic solution, the first approach for the determination of sCT in the microspheres was extraction of the peptide with an acidic saline solution. The amount of sCT recovered from three batches of HYAFF® 11 microspheres (expressed as a percentage of the theoretical or starting concentration) is shown in Table 2. Approximately 50% of the peptide was recovered by extraction with saline solution; this value was not increased by repeated extractions performed on the same sample. Assessment of the biological activity of the sCT/saline solution in rats (expressed as International Units of sCT, I.U.) showed a close correlation with the concentration measured by HLPC, confirming that the peptide recovered was biologically active.

In order to determine whether this low recovery reflected the true entrapment level of the peptide or an inefficient extraction from the microspheres, the HYAFF® 11/sCT microspheres were dissolved in DMSO and the resultant solution was analysed as before. As can be seen in Table 2, approximately 65% of the starting peptide was recovered using this method. However, although the quantity of sCT recovered from the microspheres was increased by dissolution of the microspheres in DMSO, this method presented certain practical difficulties related to the precipitation of the polymer on contact with the mobile phase. As a result, the assay of the polymer/sCT solution by HPLC was difficult to reproduce. Interestingly, following administration of sCT extracted in DMSO, the biological activity observed was 30% higher than predicted from the HPLC analysis of the sCT/DMSO solution.

These results reflected the inefficient sCT extraction with saline solution and analytical problems experienced during injection of the polymer/sCT solution into the HPLC.

A new method was developed in which the HYAFF® 11/sCT microspheres were treated with a solution of guanidine hydrochloride to reduce interactions between the peptide and the polymer and to facilitate the extraction of sCT. Using this method, the amount of sCT recovered from the HYAFF® 11 microspheres was between 75 and 80% of the starting peptide (Table 2). The sCT concentrations of the saline and DMSO extracts, measured by HPLC, were 12.1 mg/ml and 13.2 mg/ml, respectively, corresponding to 55 and 62% of the starting peptide content. The recovery of sCT from the same batch of microspheres by extraction with guanidine solution was 80%. These results confirmed that the incorporation of sCT into HYAFF® 11 microspheres was high.

In comparison, the concentration of sCT measured by the biological assay corresponded to that determined by HPLC analysis in guanidine, confirming the suitability of this latter method for the extraction and quantification of the peptide.

4. Conclusions

HYAFF® 11 microspheres containing sCT have been prepared by a solvent extraction method. By optimisation of processing parameters, small microspheres, of less than 10 mm in diameter, with smooth surfaces have been obtained. Three methods have been evaluated for the extraction and determination of sCT incorporated into the microspheres, but the extraction with a guanidine solution was the most efficient method employed. The entrapment of sCT into HYAFF® 11 microspheres was found to be high with at least 80% of the added peptide being incorporated. Furthermore, assessment of the biological activity of the peptide confirmed that the pharmacological activity of sCT was unaffected by the preparation process.

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