

Stability aspects of salmon calcitonin entrapped in poly(ether-ester) sustained release systems

R. van Dijkhuizen-Radersma^{a,*}, H.M. Nicolas^a, M. van de Weert^{b,1},
M. Blom^{a,2}, K. de Groot^a, J.M. Bezemer^{a,3}

^a *IsoTis N.V., P.O. box 98, 3720 AB, Bilthoven, The Netherlands*

^b *Department of Pharmaceutics, Faculty of Pharmacy, University of Utrecht, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*

Received 11 March 2002; received in revised form 10 August 2002; accepted 13 August 2002

Abstract

Poly(ether-ester)s composed of hydrophilic poly(ethylene glycol)-terephthalate (PEGT) blocks and hydrophobic poly(butylene terephthalate) (PBT) blocks were studied as matrix for the controlled release of calcitonin. Salmon calcitonin loaded PEGT/PBT films were prepared from water-in-oil emulsions. The initial calcitonin release rate could be tailored by the copolymer composition, but incomplete release of calcitonin was observed. FTIR measurements indicated aggregation of calcitonin in the matrix, which was not due to the preparation method of the matrices, but due to the instability of calcitonin in an aqueous environment. Release experiments showed the susceptibility of calcitonin towards the composition of the release medium, in particular to the presence of metal ions. With increasing amount of sodium ions, a decrease in the total amount of released calcitonin was observed due to enhanced aggregation. The calcitonin had to be stabilized in the matrix to prevent aggregation. Incorporation of sodium dodecyl sulphate (SDS) as a stabilizer in PEGT/PBT matrices increased the percentage of calcitonin released, but could not avoid aggregation on a longer term.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Poly(ether ester); Controlled release; Calcitonin; Stabilization; Aggregation

1. Introduction

Calcitonin (CT), a polypeptide of 32 amino acids (3500 Da) has a physiological role in the regulation of calcium homeostasis and is a potent inhibitor of osteoclastic bone resorption (Parfitt, 1987; Boden and Kaplan, 1990; Wallach et al., 1999; Lee and Sinko, 2000; Torres-Lugo and Peppas, 2000). The treatment of bone-related diseases, including Paget's disease, hypercalcemia and osteoporosis, requires frequent administration

* Corresponding author. Present address: Chienna B.V., Professor Bronkhorstlaan 10-D, 3723 MB Bilthoven, The Netherlands. Tel.: +31-30-274-0084; fax: +31-30-274-0335

E-mail address: riemke.van.dijkhuizen@chienna.com (R. van Dijkhuizen-Radersma).

¹ Present address: Department of Pharmaceutics, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen, Denmark.

² Present address: Chienna B.V., Professor Bronkhorstlaan 10-D, 3723 MB Bilthoven, The Netherlands.

³ Present address: Chienna B.V., Professor Bronkhorstlaan 10-D, 3723 MB Bilthoven, The Netherlands.

of relatively high calcitonin dosages. Different routes have been studied to deliver calcitonin more effectively, including oral (Lee and Sinko, 2000; Torres-Lugo and Peppas, 2000), nasal (Morimoto et al., 2001; Law and Shih, 2001) and vaginal (Richardson et al., 1993) delivery.

To reduce the number of doses that have to be administered, controlled release systems using biodegradable polymers are under investigation. Incorporation of the calcitonin in a polymeric matrix can protect the peptide from rapid clearance and provide a sustained release. Calcitonin release systems based on poly(lactic acid) (Asano et al., 1993; Millest et al., 1993), poly(lactic-glycolic acid) (PLGA) (Diaz et al., 1999), poly(glycolic acid) (PGA) (Lee et al., 1991) and poly(ϵ -prolactone) (PCL) (Vandamme and Gillard, 1993) have been studied so far. In this study, poly(ether ester) multiblock copolymers were investigated as potential release matrix for calcitonin. The polymers were composed of repeating blocks of hydrophilic poly(ethylene glycol) terephthalate (PEGT) and hydrophobic poly(butylene terephthalate) (PBT). These biocompatible and degradable PEGT/PBT copolymers have successfully been applied as matrix for the controlled release of proteins and peptides (Bezemer et al., 2000; van Dijkhuizen-Radersma et al., 2002). Quantitative release of fully active lysozyme has been reported from these multiblock copolymers (Bezemer et al., 2000). An important advantage of this system is that through modulation of the copolymer composition one can precisely tailor the release rate to meet therapeutic requirements. A potential additional advantage of PEGT/PBT is the presence of hydrophilic polymer chains, which may improve the stability of calcitonin (Arvinte et al., 1997; Sakuma et al., 1997).

Like many proteins and peptides (Cleland and Jones, 1996; Zhu et al., 2000), the therapeutic use of calcitonin is hampered by its physical instability (Cholewinsky et al., 1996). In aqueous solutions calcitonin has a pronounced tendency to aggregate into long, thin fibrillar aggregates, yielding a viscous and turbid dispersion (Bauer et al., 1995). Extensive research has been performed on stabilization of calcitonin in aqueous solutions (Arvinte et al., 1997; Stern and Gilligan, 1999;

Veronesi et al., 2000; Lee et al., 1992; Baudyš et al., 1996; Baudyš and Kim, 1998; Cudd et al., 1995; Zhu et al., 2000), containing organic acids surfactants, sugars, and polymers like polyvinylpyrrolidone and polyvinylalcohol. Also other solvents, like ethanol, methanol, propylene glycol and dimethyl sulfoxide, have been evaluated (Arvinte et al., 1997; Stevenson and Tan, 2000; Baudyš et al., 1996). Stability problems may also occur in sustained release systems, due to the high concentration of calcitonin in the polymer matrix. Therefore, this study focussed on the stabilization of calcitonin in the matrix. Salmon calcitonin (sCT) was used in this study because it is the most potent of the calcitonins available, well tolerated and clinically effective.

2. Materials and methods

2.1. Materials

A series of poly(ethylene glycol) terephthalate/poly(butylene terephthalate) (PEGT/PBT) copolymers were obtained from IsoTis NV (Bilthoven, The Netherlands). The poly(ether-ester) copolymers varied in PEGT/PBT weight ratio (80/20–40/60) at a constant PEG segment length (600 g mole⁻¹) and are indicated as **a**PEGT**b**PBT**c**, in which **a** is the PEG molecular weight, **b** the wt.% PEG-terephthalate and **c** (= 100-**b**) the wt.% PBT. Phosphate buffered saline (PBS), (pH 7.4) and sodium dodecyl sulphate (SDS) were purchased from Life Technologies Ltd (Paisley, Scotland). Merck (Darmstadt, Germany) supplied acetic acid (100%), sodium chloride (NaCl) and sodium dihydrogen phosphate (NaH₂PO₄). Chloroform (analytical grade), carboxymethyl cellulose sodium salt (CMC, medium viscosity) and D(-)-mannitol (extra pure) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Sigma Chemical Co (St Louis, USA) supplied Tween 80 and dextran (average mol wt. 41 272). Polyvinylalcohol (PVA, 13 000–23 000 g mole⁻¹, 87–89% hydrolyzed) and D(+)-trehalose (dihydrate 99%) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, USA). Bachum AG (Bubendorf, Switzerland) was the supplier of salmon calcitonin (sCT), which

was a gift of PowderJect Pharmaceuticals PLC (Oxford, UK).

2.2. Preparation of salmon calcitonin-loaded PEGT/PBT films

For the preparation of salmon calcitonin loaded films, an aqueous salmon calcitonin solution (0.6 ml, 20 mg ml⁻¹) was emulsified with a polymer solution (1 g PEGT/PBT in 7 ml CHCl₃) using an ultra turrax (IKA Labortechnik T25) for 30 s at 19 krpm. The resulting water-in-oil emulsion was cast on a glass plate using a casting knife. After slow evaporation of the solvent, the films were removed from the glass plate and freeze dried for at least 16 h. The resulting films had a thickness of 50–100 μm.

To study the influence of the matrix composition on the salmon calcitonin release behavior, the wt.% PEGT was varied between 40 and 80 wt.%.

The aqueous calcitonin solution was varied to evaluate the effect of the solution composition on the calcitonin release. PBS (pH 7.4) was used as the standard aqueous phase, while also experiments with acetic acid (0.0002 M, pH 4.3) and a phosphate buffer (0.1 M NaH₂PO₄, pH 4.3) as water phase were carried out. SDS was added to a acetic acid solution (0.0002 M, pH 4.3) to study the effect of this additive on the calcitonin stability.

2.3. Salmon calcitonin release from PEGT/PBT films

The salmon calcitonin release from the salmon calcitonin loaded PEGT/PBT films was investigated by incubating pieces of the films (± 1.77 cm²) in 1 ml release medium. Vials were continuously shaken at 37 °C and samples of the release medium were taken at various time points. The salmon calcitonin concentration of the buffer was determined using a micro bichinchonic acid (BCA) protein assay on an EL 312e microplate bio-kinetics reader ($\lambda = 570$ nm). The buffer was refreshed after sampling.

The standard in vitro release buffer used in this study was PBS (0.16 M Na⁺, pH 7.4). The release of calcitonin was also measured in acetic acid

(0.0002 M, pH 4.3) and a phosphate buffer (0.1 M NaH₂PO₄, pH 4.3). The influence of the presence of sodium ions in the release medium was evaluated by varying the amount of sodium chloride in water.

2.4. Infrared spectroscopy and calcitonin structure analysis

Infrared spectroscopy of calcitonin loaded and unloaded PEGT/PBT films were performed in transmission mode on a Bio-Rad FTS6000 FTIR spectrometer (Cambridge, USA), equipped with a dTGS detector. 2048 scans were averaged at 2 cm⁻¹ resolution. The calcitonin loaded film was measured in dry state directly after preparation and after incubation in PBS for 2 days at 37 °C. Calcitonin, dissolved in PBS, was measured in a transmission cell with CaF₂ windows and a 6 μm mylar[®] spacer (Graseby SpecAc, Orpington, UK). Peptide conformation was analyzed by the second derivative in the Amide I region [Susi and Byler, 1983](#)), after a 13-point Savitzky–Golay smoothing to remove white noise. For the calcitonin in PBS solution, the solvent background was first interactively subtracted to obtain a flat baseline in the region 2300–1800 cm⁻¹. Any polymer contribution to the solid state spectra was interactively subtracted to obtain a flat baseline between 1710 and 1730 cm⁻¹ ([Yang et al., 1999](#); [van de Weert et al., 2000, 2002](#)).

2.5. Screening of stabilizers to reduce calcitonin aggregation

The stability of salmon calcitonin in solution was evaluated by using UV spectroscopy ([Lee et al., 1992](#); [Baudyš et al., 1996](#); [Van de Weert et al., 2000](#)). The optical dispersion of calcitonin solutions (20 mg ml⁻¹) in PBS or acetic acid (0.0002 M, pH 4.3) was recorded on a Lambda 40 UV–Vis spectrometer (Perkin–Elmer, Norwalk, USA). Aggregation of the calcitonin was monitored by measuring the increase in turbidity at 340 nm as a function of time. The calcitonin solutions were stored at 37 °C between the measurements. Various stabilizers were evaluated for their ability to prevent calcitonin aggregation. Surface-active

compounds (Tween 80, SDS), sugars (trehalose, mannitol) and large organic molecules (CMC, PVA, dextran) were added at different concentrations to freshly prepared calcitonin (20 mg ml^{-1}) solutions in PBS or acetic acid (0.0002 M , pH 4.3).

3. Results and discussion

3.1. Calcitonin release in PBS from PEGT/PBT films

Calcitonin release kinetics from PEGT/PBT films were first evaluated as a function of copolymer composition. Previous studies with vitamin B₁₂ (1355 D), showed a constant release over 12 weeks from PEGT/PBT copolymers with 300 g mole^{-1} PEG-segments (van Dijkhuizen-Radersma et al., 2002). These copolymer compositions had a very low equilibrium swelling ratio. Being three times the size of vitamin B₁₂, salmon calcitonin release would require more swollen matrices to allow diffusion. Therefore, copolymers with PEG-segment lengths of 600 g mole^{-1} were selected for this study. The PEGT/PBT copolymers varied between 40 and 80 wt.% PEGT. A higher wt.% PEGT resulted in a higher swelling of the matrix (Bezemer et al., 2000; van Dijkhuizen-Radersma et al., 2002).

The copolymer composition determined the calcitonin release in PBS to a large extent (Fig.

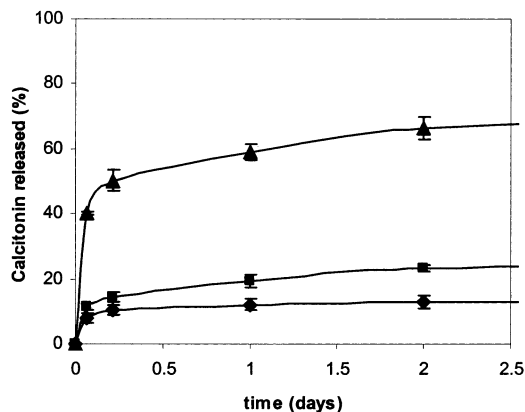


Fig. 1. Salmon calcitonin release from 600PEGT40PBT60 (●), 600PEGT60PBT40 (■) and 600PEG80PBT20 (▲) films in PBS ($n = 3$; \pm Standard Deviation (S.D.)).

1). Sixty percent of the encapsulated calcitonin was released within 2 days for the 600PEGT80PBT20 film, whereas a slower initial release was observed for the copolymers with lower wt.% PEGT. For all compositions, the calcitonin release was incomplete. The slower release of calcitonin from 600PEGT60PBT40 and the 600PEGT40PBT60 copolymers can be explained by the lower degree of swelling of the polymer matrix.

The slow diffusion through the PEGT/PBT matrix does not explain the observed incomplete calcitonin release (Fig. 1). Possibly, calcitonin aggregation has occurred in the matrices, either during the release experiments or during the preparation process. FTIR measurements have been performed on calcitonin loaded PEGT/PBT films to investigate this further. Bauer et al. already showed that FTIR spectroscopy provides a sensitive analytical tool to monitor aggregation phenomena of calcitonin in aqueous solutions (Bauer et al., 1994).

3.2. Integrity of calcitonin in PEGT/PBT matrix

Calcitonin loaded films and aqueous calcitonin solutions were analyzed by FTIR to detect conformation changes of calcitonin. FTIR analysis of the calcitonin-loaded films was somewhat hampered by the low amount of calcitonin in the film. Within that limitation, the FTIR spectrum did not indicate any obvious change in secondary structure of calcitonin directly after preparation of the films (Fig. 2). However, when the film was incubated for 2 days in PBS, the secondary structure of the calcitonin remaining in the film was markedly different. A clear band at 1630 cm^{-1} is observed, which is typical of intermolecular β -sheet formation (non-covalent aggregation). A calcitonin solution stored for 20 h at room temperature also indicated the early onset of aggregation (Fig. 2). It is well-known that calcitonin is unstable upon incubation in aqueous solution (Wallach et al., 1999; Morimoto et al., 2001; Law and Shih, 2001; Cholewinsky et al., 1996). Bauer et al. reported an increase in time for both α and β -contents in human calcitonin solutions determined by ATR-FTIR (Bauer et al., 1994). Arvinte et al. related these α helical and β -sheet

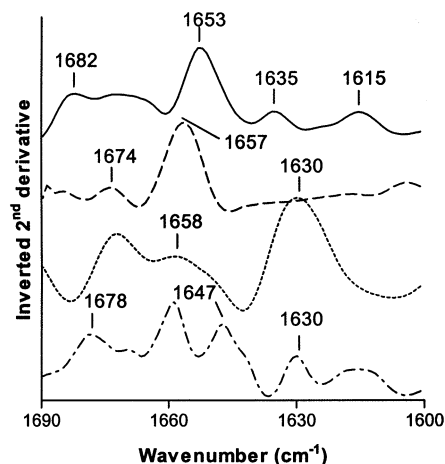


Fig. 2. Inverted second derivative spectra in the Amide I region of calcitonin in solution (solid line), in a 600PEGT60PBT40 film (dashed line) directly after preparation, in a 600PEGT60PBT40 film (dotted line) after 1 day incubation in PBS at 37 °C, and in solution after 1 day incubation at room temperature (dash-dotted line).

components to the fibrillated state of aggregated calcitonin (Arvinte et al., 1993). Thus, the aggregation of calcitonin in PEGT/PBT films is most likely caused by incubation in solution, rather than by the preparation method.

Cholewinsky et al. have described several degradation pathways of calcitonin in aqueous solutions (Cholewinsky et al., 1996). Besides pH and temperature, the presence of metal ions is known to facilitate aggregation of calcitonin. The PEGT/PBT films described above contained a significant amount of sodium ions, as the calcitonin was incorporated in these films as a solution in PBS. In addition, the release experiments have been carried out in PBS as release medium as well. To study the effect of the presence of sodium ions on the release of calcitonin from PEGT/PBT films, the composition of both the incorporated aqueous calcitonin solution as well as the release medium have been varied.

3.3. Effect of sodium ions on calcitonin release

For the release experiments described below the 600PEGT60PBT40 copolymer was selected. The release of calcitonin was measured as a function of

the inner phase and release buffer compositions. Either a 0.1 M phosphate or a 0.0002 M acetic acid buffer was used, both formulated at pH 4.3 to exclude pH effects. It is known that calcitonin is less prone to aggregation at acidic pH (Lee et al., 1992) in acetic acid solutions (Arvinte et al., 1997; Baudyš et al., 1996). As shown in Fig. 3, variation of the composition of the release buffer composition has a major influence on the calcitonin release. Using phosphate buffer as release medium, the total calcitonin release is only 20–25%, whereas acetic acid buffer results in a release of 80–100%. Variation of the inner phase composition has much less influence on the total amount of calcitonin released, with acetic acid buffer as inner phase yielding a slightly higher total release than phosphate buffer. This suggests that the calcitonin release profile is mainly determined by the release medium composition. The complete release in the acetic acid release medium indicated that no aggregation of the calcitonin had occurred, possibly due to the absence of metal ions.

The effect of the sodium ions in the release medium on the calcitonin release was evaluated in more detail. Samples of the calcitonin loaded films were incubated in aqueous solutions containing various sodium chloride concentrations. In water without sodium chloride, the release was complete within 2 days (Fig. 4). With increasing sodium concentration, however, the calcitonin release

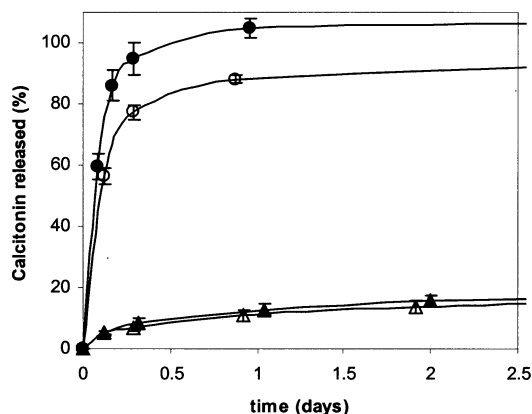


Fig. 3. Salmon calcitonin release from 600PEGT60PBT40 films with phosphate buffer (\blacktriangle) and acetic acid (\bullet) as inner phase. Release medium: 0.1 M NaH_2PO_4 (open symbols) and 0.0002 M acetic acid (filled symbols) ($n = 3$; \pm S.D.).

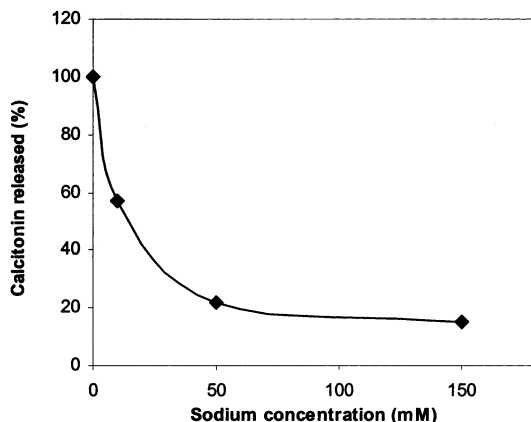


Fig. 4. Effect of sodium ions in release medium on the total amount of salmon calcitonin released from 600PEGT60PBT40 films within 2 days.

became incomplete, indicating calcitonin aggregation in the matrix (Fig. 4). In a release medium containing 150 mM sodium chloride, only 15% of the calcitonin was released from 600PEGT60PBT40 matrices. Apparently, the sodium ions had diffused from the release buffer into the polymer matrix, inducing calcitonin aggregation. As the sodium ion concentration in blood is 142 mM, the calcitonin should be stabilized in the matrix to avoid aggregation.

3.4. Effect of additives on calcitonin stability and long term release

To be able to select the proper stabilizer for calcitonin in the matrix, several additives have been investigated in solutions of calcitonin in PBS and acetic acid. The samples were checked periodically for the presence of a precipitate, a gel or turbidity, which are indicative of fibrillation (Arvinte et al., 1993). Table 1 shows the effect on the stability of calcitonin solutions with several additives. Sodium dodecyl sulphate (SDS) was the only additive that showed a long-term stabilizing effect. Therefore, the effect of SDS was studied in more detail in both PBS and acetic acid (Table 2). By increasing the concentration of the SDS from 0 to 2% (w/v), the stability of the calcitonin solutions varied. Surprisingly, the stability of the solutions decreased with increasing SDS concentration up to

Table 1
Stability of salmon calcitonin (20 mg ml^{-1}) in PBS with several additives

Additive	Concentration (% w/v)	Stabilization period ^a
–	–	2 h
Trehalose	1	4 h
Mannitol	1	< 1 h
SDS	1	5 days
Tween 80	1	6 h
PVA	1	< 1 h
Dextran	1	6 h
CMC	0.5	< 1 h

^a Time period in which no turbidity change was observed.

Table 2
Stability of calcitonin solution (20 mg ml^{-1}) in PBS or 0.0002 M acetic acid at various SDS concentrations

SDS (% w/v)	Buffer	Stabilization period ^a
0	PBS	2 h
0.24	PBS	1 h
0.5	PBS	< 1 h
1.0	PBS	5 days
2.0	PBS	> 2 months
0	Acetic acid	> 2 months
0.24	Acetic acid	2 h
0.5	Acetic acid	< 1 h
1.0	Acetic acid	> 2 months
2.0	Acetic acid	> 2 months

^a Time period in which no turbidity change was observed.

0.5% (w/v), while the stability increased at higher SDS concentrations. Baudyš et al. attributed the stabilizing effect of SDS to the formation of a very stable complex between calcitonin and SDS in SDS micelles (Baudyš et al., 1996; Baudyš and Kim, 1998). Although the critical micelle concentration value for SDS is 0.24%, we only observed a long-term stabilizing effect at SDS concentrations of 1% and higher. This might be due to the relatively high calcitonin concentration used in our study. Possibly, at lower SDS concentrations, the number of potential calcitonin binding sites in SDS micelles was exhausted due to the high calcitonin concentration (Baudyš and Kim, 1998). Partial stabilized calcitonin molecules apparently aggregate even faster than unstabilized calcitonin (no SDS).

Compared with the PBS solutions, an increased calcitonin stability was already observed for acetic acid containing solutions in the absence of SDS. Several other groups, therefore, have added acetic acid to stabilize calcitonin in solution (Arvinte et al., 1997; Baudyš et al., 1996; Baudyš and Kim, 1998; Cudd et al., 1995). In our release experiments described above, we also observed a (small) positive effect of acetic acid in the inner phase on the total amount of calcitonin released (Fig. 3). Therefore, acetic acid was chosen as inner phase to evaluate the effect of SDS on the sustained release of calcitonin from PEGT/PBT films. The release from 600PEGT60PBT40 films, containing 0, 1 and 2% SDS in the inner phase, has been measured in PBS. Fig. 5 shows an increase in total amount of calcitonin released with increasing SDS concentration. However, similar to the PEGT/PBT matrices without SDS (Fig. 1), the release stops after 2 days, indicating that the calcitonin has aggregated during the release period. Apparently, the SDS concentration was not high enough to fully stabilize the calcitonin in the matrix. This could be either due to the diffusion of SDS out of the matrix during the release experiment, or due to the high concentration of calcitonin in the matrix compared with the solutions used in the turbidity experiment. The increasing amount of calcitonin released with increasing SDS concentration may be the result of the stabilizing effect of the SDS on

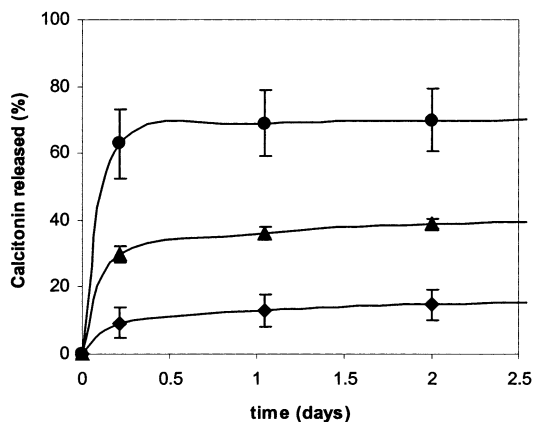


Fig. 5. Salmon calcitonin release from 600PEGT60PBT40 films with acetic acid as inner phase containing 0% (◆), 1% (▲) and 2% (●) of SDS (w/v). Release in PBS ($n = 3$; \pm S.D.).

the calcitonin, but it could also be due to a more open matrix structure. The presence of SDS in the water-in-oil emulsion may destabilize the emulsion, resulting in more open matrix, which in turn will increase the calcitonin diffusion rate. Consequently, a larger amount of calcitonin will be released before aggregation has started.

4. Conclusion

In order to obtain a sustained release system for salmon calcitonin, calcitonin loaded PEGT/PBT films were prepared from water-in-oil emulsions. Although the initial calcitonin release rate could be tailored by the copolymer composition, no complete release of calcitonin was observed. FTIR measurements indicated aggregation of calcitonin in the matrix, which was not due to the preparation method of the matrices, but due to the instability of calcitonin in an aqueous environment. Release experiments showed the susceptibility of calcitonin towards the release medium, in particular to the presence of metal ions in the release medium. With increasing amount of sodium ions, a decrease in the total amount of released calcitonin was observed due to aggregation. The calcitonin had to be stabilized in the matrix to prevent aggregation. Comparison of several additives in absorption experiments showed that sodium dodecyl sulphate (SDS) had a long-term stabilizing effect on calcitonin in solution. Incorporation of SDS in PEGT/PBT matrices indeed increased the percentage of calcitonin released, but still no sustained release of calcitonin was obtained.

Further experiments will focus on the stabilizing calcitonin in the matrix by incorporation of other additives. In addition, the activity of calcitonin after release from PEGT/PBT matrices will be investigated by in-vivo experiments.

References

- Arvinte, T., Cudd, A., Drake, A.F., 1993. The structure and mechanism of formation of human calcitonin fibrils. *J. Biol. Chem.* 268, 6415–6422.

- Arvinte, T., Cudd, A., Philips, J., 1997. Fibrillated calcitonin pharmaceutical compositions. US patent 5 593 962, 14 January.
- Asano, M., Yoshida, M., Omichi, H., Mashimo, T., Okabe, K., Yuasa, H., Yamanaka, H., Morimoto, S., Sakakibara, H., 1993. Biodegradable poly(DL-lactic acid) formulations in a calcitonin delivery system. *Biomaterials* 14, 797–799.
- Baudyš, M., Mix, D., Kim, S.W., 1996. Stabilization and intestinal absorption of human calcitonin. *J. Control. Rel.* 39, 145–151.
- Baudyš, M., Kim, S.W., 1998. Stabilisation and oral delivery of calcitonin. US patent 5 726 154, 10 March.
- Bauer, H.H., Müller, M., Goette, J., Merkle, H.P., Fringeli, U.P., 1994. Interfacial adsorption and aggregation associated changes in secondary structure of human calcitonin monitored by ATR-FTIR spectroscopy. *Biochemistry* 33, 12276–12282.
- Bauer, H.H., Aebi, U., Häner, M., Hermann, R., Müller, M., Merkle, H.P., 1995. Architecture and polymorphism of fibrillar supramolecular assemblies produced by in vitro aggregation of human calcitonin. *J. Struct. Biol.* 115, 1–15.
- Bezemer, J.M., Radersma, R., Grijpma, D.W., Dijkstra, P.J., Feijen, J., Van Blitterswijk, C.A., 2000. Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices. *J. Control. Rel.* 64, 179–192.
- Boden, S.D., Kaplan, F.S., 1990. Calcium homeostasis. *Orthopedic Clin. North Am.* 21, 31–42.
- Cholewinsky, M., Lückel, B., Horn, H., 1996. Degradation pathways, analytical characterization and formulation strategies of a peptide and a protein Calcitonin and human growth hormone in comparison. *Pharm. Acta Helv.* 71, 405–419.
- Cleland, J.L., Jones, A.J.S., 1996. Stable formulations of recombinant human growth hormone and interferon- γ for microencapsulation in biodegradable microspheres. *Pharm. Res.* 13, 1464–1475.
- Cudd, A., Arvinte, T., Gaines Das, R.E., Chinni, C., MacIntyre, I., 1995. Enhanced potency of human calcitonin when fibrillation is avoided. *J. Pharm. Sci.* 84, 717–719.
- Diaz, R.V., Llabrés, M., Évora, C., 1999. One-month sustained release microspheres of ^{125}I -bovine calcitonin, in vitro–in vivo studies. *J. Control. Rel.* 59, 55–62.
- Law, S.L., Shih, C.L., 2001. Characterization of calcitonin-containing liposome formulations for intranasal delivery. *J. Microencap.* 18, 211–221.
- Lee, K.C., Soltis, E.E., Newman, P.S., Buron, K.W., Mehta, R.C., DeLuca, P.P., 1991. In vivo assessment of salmon calcitonin sustained release from biodegradable microspheres. *J. Control. Rel.* 17, 199–206.
- Lee, K.C., Lee, Y.J., Song, H.M., Chun, C.J., DeLuca, P.P., 1992. Degradation of synthetic salmon calcitonin in aqueous solution. *Pharm. Res.* 9, 1521–1523.
- Lee, Y.-H., Sinko, P.J., 2000. Oral delivery of salmon calcitonin. *Adv. Drug Deliv. Rev.* 42, 225–238.
- Millest, A.J., Evans, J.R., Young, J.J., Johnstone, D., 1993. Sustained release of salmon calcitonin in vivo from lactide:glycolide copolymer depots. *Calcif. Tissue Int.* 52, 361–364.
- Morimoto, K., Katsumata, H., Yabuta, T., Iwanaga, K., Kakemi, M., Tabata, Y., Ikada, Y., 2001. Evaluation of gelatin microspheres for nasal and intramuscular administrations of salmon calcitonin. *Eur. J. Pharm. Sci.* 13, 179–185.
- Parfitt, A.M., 1987. Bone and plasma calcium homeostasis. *Bone* 8, S1–S8.
- Richardson, J.L., Miglietta, M., Ramires, P.A., Rochira, M., Fisher, A.N., Farraj, N.F., Illum, L., Benedetti, L.M., 1993. Vaginal administration of calcitonin in rats and sheep using microspheres of hyaluronane esters. *Proceedings of the International Symposium on Controlled Release Bioactive Material*, 20.
- Sakuma, S., Ishida, Y., Sudo, R., Suzuki, N., Kikuchi, H., Hiwatari, K., Kishida, A., Akashi, M., Hayashi, M., 1997. Stabilization of salmon calcitonin by polystyrene nanoparticles having surface hydrophilic polymeric chains, against enzymatic degradation. *Int. J. Pharm.* 159, 181–189.
- Stern, W., Gilligan, J.P., 1999. Oral salmon calcitonin pharmaceutical products. US patent 5 912 014, 15 June.
- Stevenson, C.L., Tan, M.M., 2000. Solution stability of salmon calcitonin at high concentration for delivery in an implantable system. *J. Peptide Res.* 55, 129–139.
- Susi, H., Byler, D.M., 1983. Protein structure by fourier transform infrared spectroscopy: second derivative spectra. *Biochem. Biophys. Res. Commun.* 115, 391–397.
- Torres-Lugo, M., Peppas, N.A., 2000. Transmucosal delivery systems for calcitonin: a review. *Biomaterials* 21, 1191–1196.
- Vandamme, T.F., Gillard, J.L., 1993. Poly- ϵ -caprolactone microspheres containing calcitonin: preparation, morphology and release properties. *Proceedings of the International Symposium on Controlled Release and Bioactive Material*, 20.
- Van de Weert, M., Van't Hof, R., Van der Weerd, J., Heeren, R.M.A., Posthuma, G., Hennink, W.E., Crommelin, D.J.A., 2000. Lysozyme distribution and conformation in a biodegradable polymer matrix as determined by FTIR techniques. *J. Control. Rel.* 68, 31–40.
- Van de Weert, M., Van Dijkhuizen-Radersma, R., Bezemer, J.M., Hennink, W.E., Crommelin, D.J.A., 2002. Reversible aggregation of lysozyme in a biodegradable amphiphilic multiblock copolymer. *Eur. J. Pharm. Biopharm.* 54, 89–93.
- Van Dijkhuizen-Radersma, R., Péters, F.L.A.M.A., Stienstra, N.A., Grijpma, D.W., Feijen, J., De Groot, K., Bezemer, J.M., 2002. Control of vitamin B12 from poly(ethylene glycol)/poly(buthylene terephthalate) multiblock copolymers. *Biomaterials* 23, 1527–1536.
- Veronesi, P.A., Peschechera, E., Veronesi, A.M., 2000. Pharmaceutical non inorganic saline solutions for endonasal administration of a calcitonin. US patent 6 087 338, 11 July.
- Wallach, S., Rousseau, G., Martin, L., Azria, M., 1999. Effects of calcitonin on animal and in vitro models of skeletal metabolism. *Bone* 25, 509–516.

Yang, T.-H., Dong, A., Meyer, J., Johnson, O.L., Cleland, J.L., Carpenter, J.F., 1999. Use of infrared spectroscopy to assess secondary structure of human growth hormone within biodegradable microspheres. *J. Pharm. Sci.* 88, 161–165.

Zhu, G., Mallery, S.R., Schwendeman, S.P., 2000. Stabilization of proteins encapsulated in injectable poly(lactide-co-glycolide). *Nat. Biotechnol.* 18, 52–56.