ULTRASONIC ATOMISATION: A NOVEL METHOD FOR MICROENCAPSULATION OF PROTEINS AND PEPTIDES

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Running title: Ultrasonic atomisation to produce microparticles

J. controlled release

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SUMMARY

INTRODUCTION

New carriers for peptide and protein based drugs which release the highly active compound slowly and controlled have already been studied and tested. One of the most successful carriers are poly-lactide-co-glycolide polymer (PLGA), or poly lactic polymer (PLA) microspheres (1). The encpasulation may protect the protein and peptide from enzymatic degradation and extend their release over a prolonged time span.

Although the preparation methods of PLGA microspheres have been well established, the choice of the techniques to prepare protein and/or peptide loaded microspheres is complicated due to the potentially detrimental effect of several formulation and process parameters, such as type of organic solvent, shear forces, surface-surface interactions, and temperature has to be considered.

Due to low resistance against higher temperatures, spray-drying of peptides and proteins is very critical. Only very stable protein may be encapsulated with this method.

Another very common microencapsulation method, solvent evaporation, shows on one hand a relative low encapsulation efficiency for water soluble drugs and on the other hand, due to the emulsification process (often double emulsion) proteins and peptides may interact with and aggregate on interfaces.

The aim of this study was the establishment of a novel method based on ultrasonic atomisation for the microencapsulation of proteins and peptides as an easy to use and to scale-up, single step microencapsulation method. For this, three different types of peptide/protein were used. First, as a very small tetrapeptide, thymocartin (TP4, Arg-Lys-Asp-Val), a bioac-

tive fragment of the naturally occuring thymus hormone thymopoietin, which has shown potential for the treatment of immunodeficiency diseases, as a very water soluble peptide was used. Second, an octapeptide, vapreotide pamoate, a somatostatin analogue was used as a water insoluble peptide derivative. And last but not least, bovine serum albumin (BSA) was used as a moderate water-soluble model protein.

MATERIALS AND METHODS

The biodegradable end-group capped poly(D,L-lactide) PLA (Resomer® R 202), end-group capped and uncapped poly(D,L-lactide-co-glycolide) PLGA 50:50 (Resomer® RG 502 and Resomer® RG 502 H) were purchased from Boehringer Ingelheim (Ingelheim, Germany). Bovine serum albumin (BSA) was supplied by Serva (Heidelberg, Germany). The somatostatin analogue vapreotide pamoate RC-160 (D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH_2) was synthesised by Novabiochem (Laufingen, Switzeland). Foetal bovine serum was obtained from Gibco BRL (Basel, Switzerland). Thymocartin (TP4, Arg-Lys-Asp-Val-NH_2) was obtained from Fluka AG (Buchs, Switzerland). Octamethylcyclotetrasiloxan (OMCTS) was a gift from Th. Goldschmidt (Essen, Germany). All other materials were of analytical or pharmaceutical grade (from Fluka AG).

Microsphere preparation technology

The principle of the microsphere preparation technology proposed here consisted in ultrasonic spraying of a polymer solution, containing a drug, into a non-solvent for the polymer, such as OMCTS (Fig. 1).

PLA / PLGA microspheres were typically prepared by feeding the polymer solution at a rate of 2 ml×min^{-1} through the ultrasonic atomiser Vibracell VC 54 AT (Sonics & Materials, Danbury, CT, USA) and hardening the droplets in 200 ml OMCTS contained in a 1000 ml

flask. The OMCTS-phase was stirred at 300 rpm using an propeller stirrer for 3h to obtain solid microparticles.

For microencapsulating BSA and TP4, the protein, or peptide, respectively was dissolved in water, at an amount corresponding to a protein:polymer ratio of 2% (w/w). The polymer PLA was dissolved in ethylformate, to obtain a 2% and 20% (w/w) polymer solution, respectively. Aqueous protein / peptide- and organic polymer solutions were then mixed and ultrasonicated to form a W/O-emulsion. This emulsion was then atomised as described above.

Vapreotide pamoate was dissolved in a water / propanol mixture (molecular ratio of 0.2), at an amount corresponding to a peptide:polymer ratio of 10% (w/w). The polymers PLGA 50:50H and PLGA 50:50 were dissolved in ethylformate to obtain a 10% polymer solution. The two solutions were mixed and then atomised as described above.

The hardened microparticles were separated from the OMCTS by passing the mixture through a 0.8 μm cellulose acetate membrane filter (Sartorius, Germany).

The particles were then washed with 0.1% (*w/v*) poloxamer 188 solution (Synperonic® F-68, ICI, Middlesborough, UK) to remove unencapsulated drug, followed by washing with pure water, collected on a 0.8 μm cellulose acetate membrane filter and then dried under vacuum (<20 mbar) for 24 h.

Particle morphology and size distribution

Shape and surface characteristics of the microparticles were examined in a scanning electron microscope (Hitachi, Switzerland). Particle sizes were determined by laser light scattering (MasterSizer X version 1.2a, Malvern Instruments, Malvern, UK). Typically, approx. 10 mg microspheres were carefully pounded in a mortar with a few drops of polysorbate 20, followed by addition of 2 ml distilled water. The particle dispersion was then transferred into a small volume presentation unit (Malvern) and ultrasonicated for 30 seconds at output 40

(Vibracell, VC50T, Sonics and Materials, Danburry, USA). The average particle size was expressed as volume mean diameter in µm.

Encapsulation efficiency and burst release

BSA and TP4 loading of the microspheres was determined by dissolving approx. 20 mg of the loaded particles in methylene chloride, recovering the undissolved protein on a 0.2 µm regenerated cellulose filter (RC 58, Schleicher and Schuell, Dassel, Germany) and eluting the protein, or peptide, respectively, from the filter with 67 mM phosphate buffered saline (PBS) of pH 7.4. The BSA content was determined by fluorescence spectroscopy using excitation and emission wavelengths of 277 and 339 nm, respectively (Fluromax™, Spex, Edison, NJ, USA). All determinations were made in triplicate. TP4 content was determined with HPLC as described below.

The vapreotide content in the PLGA microspheres was determined by HPLC according to a method described previously (2). The peptide loaded microspheres were dissolved in 3 ml of acetonitrile, to which 2 ml of chloroform were added, and the mixture was stirred vigorously. The peptide was extracted three times with 2 ml of triethylamino phosphate buffer of pH 2.3.

The entrapment efficiency of encpasulated protein / peptide was calculated as the ratio of actually measured to theoretical (nominal) drug content in the microspheres.

The *in vitro* burst and long time release profile for BSA and TP4 was determined by suspending approx. 30 mg microspheres in 5 ml of 67 mM PBS (phosphate buffered saline), pH 7.4, containing 0.02% sodium azide in borosilicate vials. The vials were placed in a rotating chamber at 37°C. At given time intervals, three vials for each microsphere formulation were withdrawn, 200 µl sample were replaced with the same amount of fresh buffer and the amount of BSA released was assayed fluorimetrically as specified above and TP4 was assayed with the HPLC method described below. All determinations were made in triplicate.

In vitro drug release profiles for the vapreotide (up to 28 days) were obtained by incubating the microspheres (approx. 10 mg accurately weighed, n=3) in 4.0 ml of serum preserved with 0.02% (w/w) of thiomersal. Incubation took place in rotating vials at 37 °C. Due to the instability of the peptide in the release medium (2), the amount of drug released was determined indirectly by measuring the amount of drug remaining in the microspheres. At given time intervals, three vials for each microsphere formulation were withdrawn, and the polymer particles were separated by centrifugation. After removing the supernatant, the polymer mass was dried overnight at 10 mbar and room temperature. The amount of remnant vapreotide in the microspheres was determined by HPLC, as described below. The peptide extracted from the incubated microspheres was intact, as no degradation products were detected in the extraction solution with the sensitive HPLC-method used (2).

HPLC-method for peptide assay

Vapreotide Pamoate

The intact peptide was analysed by HPLC (Column Licrospher® RP-18, 4 x 250 mm, Merck, Darmstadt, Germany). The elution phase consisted of a gradient of A (triethylammonium phosphate buffer of pH 2.3; TEAP) and B (acetonitrile/TEAP pH 2.3, 60/40), with B increasing from 30 to 80% (v/v) within 25 min. Detection was at 215 nm.

TP4

The concentrations of TP4 were mesured with a HPLC method described elsewhere (3). Briefly, intact peptide and metabolite concentrations were determined by RP-HPLC following centrifugation. The set-up consisted of an autosampler AS-4000, gradient pump L-6200A, UV-VIS detector L-4250 (all from Merck, Darmstadt, Germany).

TP4 was analysed using a LiChrospher 100 RP-8 column (5mm, 250 x 4 mm, Merck) as a stationary phase and an application of a gradient program: : Solvent A was a mixture of 25% (V/V) acetonitrile and 75% (V/V) of 0.1% ortho-phosphoric acid containing 0.01 M oc-

tane-1-sulfonic acid and solvent B was a mixture of 35% (V/V) acetonitrile and 65% (V/V) of 0.1% ortho-phosphoric acid containing 0.01 M octane-1-sulfonic acid. A linear gradient from 100% to 0% A and from 0% to 100% B in 5 minutes was used, followed by 100% B at a flow rate of 1.5 ml/min. Detection was by UV absorbance measurement at a wavelength of 202 nm.

In vivo study

Two particular microspheres preparations (the formulations encapsulating vapreotide) were tested in male Sprague-Dawley rats (weighing 380-400 g, from C.E.R.J., Les Genest St. Isle, France.). The animals were maintained under constant environmental conditions (22 ± 1°C; 50 ± 5% relative humidity). Food and water were available *ad libitum*. Microspheres were suspended in an injection vehicle (the dose of peptide per rat was 1.5 mg, calculated as vapreotide base) and administered intramuscularly. Blood samples were collected at different time intervals for 28 days. Vapreotide concentrations in the blood samples were determined by radio-immuno assay (4) with a sensitivity of 50 pg/ml.

RESULTS

Particle morphology and size distribution

Fig.2 and Tab.1 show PLGA 50:50H microparticles with a volume weighed mean diameter of xxx μm and a smooth and non-porous surface. Tab.1 indicates also that a 2% polymer solution lead to much smaller particles than a 20% polymer solution. This can be readily explained by the differences in viscosity.

Entrapment efficiency

BSA

The encapsulation efficiency of BSA into PLA microspheres 25.6% for the microspheres prepared with a 2% polymer solution and 24.1% for the preparation made with 20% polymer solution. The two microsphere preparations made from 2% polymer solutions of PLGA 50:50 and PLGA 50:50H were leading in encapsulation efficiencies of 22.6% and 18.6%, respectively. These results were quiet similar to other microsphere preparation methods. Preparations with 20% PLGA 50:50 and 50:50H solutions didn't lead to any microspheres due to high tendency to aggregate during the hardening process.

Vapreotide pamoate

The highest entrapment efficiency of 92.7% was achieved with the encapped polymer (RG 502H), while only 63.3% efficiency was achieved with the capped polymer (RG 502). This results agree with observations by Blanco et al. [3].

TP4

In case of the very water soluble peptide TP4 the encapsulation efficiencies were comparable to other methods xxxLit. Only 20% of peptide were found in the microparticles made with a 2% polymer solution and 33% in the particles made with a 20% polymer solution.

Burst release

Table 2 shows the burst releases of the different formulations, which are comparable to other methods used for microencapsulation of proteins and peptides. It is obvious that formulations made with 2% polymer solution are showing a higher burst release due to the larger surface area compared to the formulations made from 20% polymer solutions. On the other hand, PLGA 50:50H microspheres show a higher burst release because of their more extended swelling due to their uncapped hydrophilic endgroups.

In vitro release kinetics

BSA

Other studies showed similar release profiles for BSA loaded PLA microparticles. The results implied that due to the large surface area the formulation made with 2% polymer solution release the BSA a lot faster (also with a higher burst release, see Tab. 2) than the formulation made with 20% polymer concentration.

RC-160

In a previous study (2), the *in vitro* release of vapreotide pamoate from microspheres has been shown to depend greatly on the type of release medium. Serum was found to be the most appropriate medium that produced the best agreement with in vivo data. For this reason, serum, preserved with 0.02% of thiomersal, was used here again as release medium.

Figure 4 depicts the *in vitro* release profiles of vapreotide from microspheres made of end-group uncapped PLGA 50:50 (RG502H) and end-group capped PLGA 50:50 (RG502) with nominal drug loadings of 10%. The RG 502H microparticles released the peptide faster (72.5% in 28 days) than the RG 502 microparticles (66.9% in 28 days). Also, the burst release was much lower with the capped polymer (5.4%) compared to the uncapped one (29.7%) after 6 hours (Tab. 1). This was probably due to a greater polarity attributed by the presence of more polar end groups in the RG 502H polymer which resulted in a more rapid hidratation of the microspheres. These observations agree with results published by others [4].

TP4

Due to the high water solubility, TP4 is released very fast from the microparticles (also with a very high burst release, see also Tab. 2). The reason for the uncomplete release (only up to 80% was released) is due to the relative instability of the peptide in an aqueous phase such as the release medium (3).

In vivo study

For the pharmacokinetic study in rats, end-group uncapped PLGA 50:50 microspheres and end-group capped PLA microspheres with 10% nominal loading of vapreotide, were selected. Each formulation was injected i.m. into rats, and vapreotide serum concentrations monitored for 28 days (Fig. 6). Microspheres produced with the uncapped polymer showed therapeutic drug levels (1 ng/ml) for up to at least 10 days. By contrast, the microspheres made with the Resomer RG 502, produced detectable serum levels for one day only. In addition, we observed very modest burst release with both formulations.

DISCUSSION

During this work we were able to demonstrate that ultrasonic atomisation is suitable and very promising for the microencapsulation of protein and peptides. Especially the poorly water soluble vapreotide pamoate showed a very efficiently microencapsulation into end-capped and uncapped PLGA 50:50 polymers by this method. On could remark that this was already shown with spray-drying (2), but one of the biggest advantages of this novel method is the easy set-up, scale-up and the possibility to the microspheres produce aseptically without a cost-intensive equipment (5). However, (2) showed that gamma-irradiation had only minor effect on the pharmacokinettic and in vitro release profiles of vapreotide pamoate. Still, the advantage of easily aseptically produced microparticles compared to γ-irradiation of the final product, which still contains the risk of polymer and/or drug degradation with creating of toxic products.

On the other hand, BSA containing microparticles showed “normal” characteristics as found with other preparation methods. And the microparticles loaded with TP, the very water soluble peptide, showed, not very surprisingly, a high burst release and fast (long time) release. But also for these two, not very enthusiastic formulations the advantage of of this novel

method and its easy set-up, scale-up and the possibility to the microspheres produce aseptically without a cost-intensive equipment weights heavily.

In conclusion, we were able to show that the ultrasonic atomisation is suitable to produce microspheres reproducible and safe, and we were able to obtain microspheres with very promising characteristics.

LITERATURE

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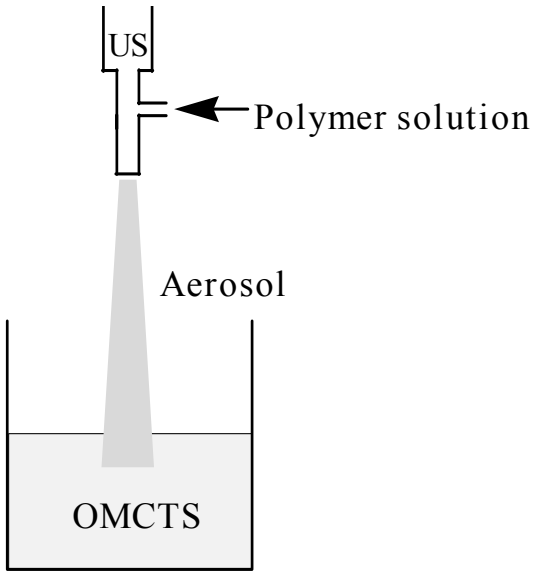

Fig. 1: Schematic illustration of the experimental set-up for preparing PLA / PLGA microspheres by ultrasonic (US) atomisation. OMCTS: octamethylcylcotetrasiloxane

Fig.2:

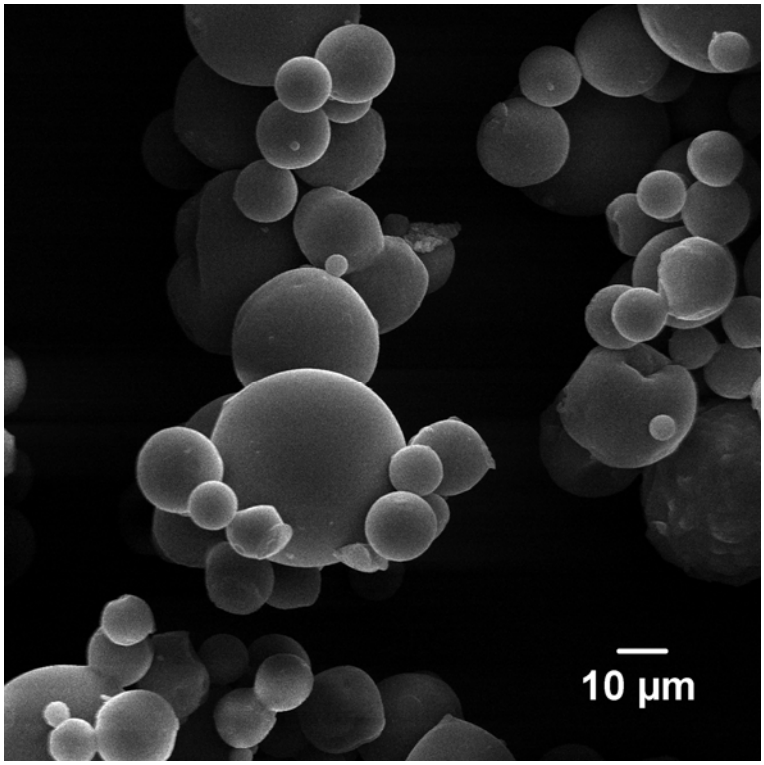

Fig.2: RC-160 loaded PLGA 50:50H microspheres. The electron micrographs confirmed the results obtained from the mastersizer (Bar indicates 10µm).

Fig. 3:

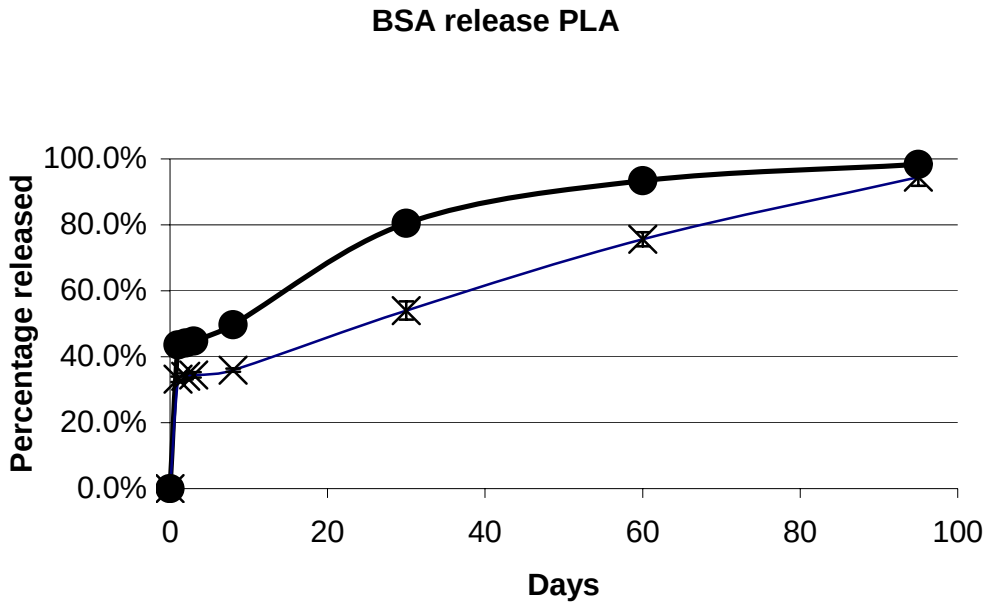

Fig.3: Release kinetics of BSA from microparticles made from 2% (●) and 20% (×) polymer solutions of PLA 202

Fig.4:

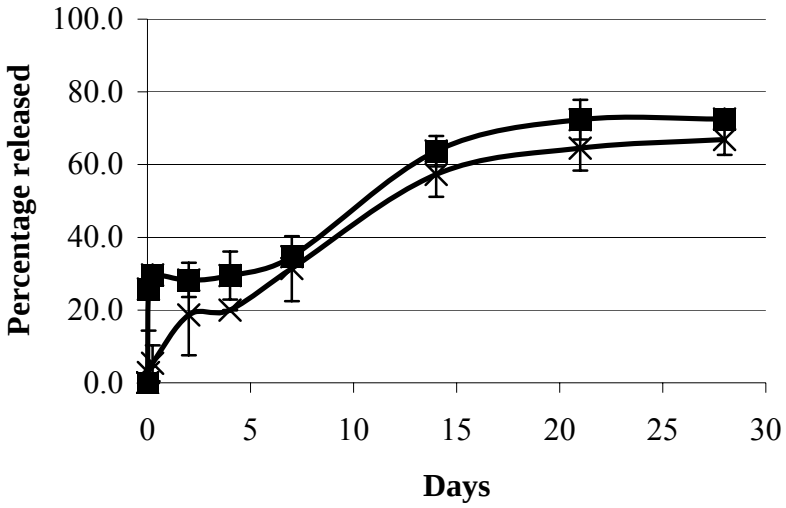

Fig.4: Release kinetics of RC-160 from microparticles made from 10% polymer solutions of PLGA 50:50 (×) and PLGA 50:50H (■).

Fig.5:

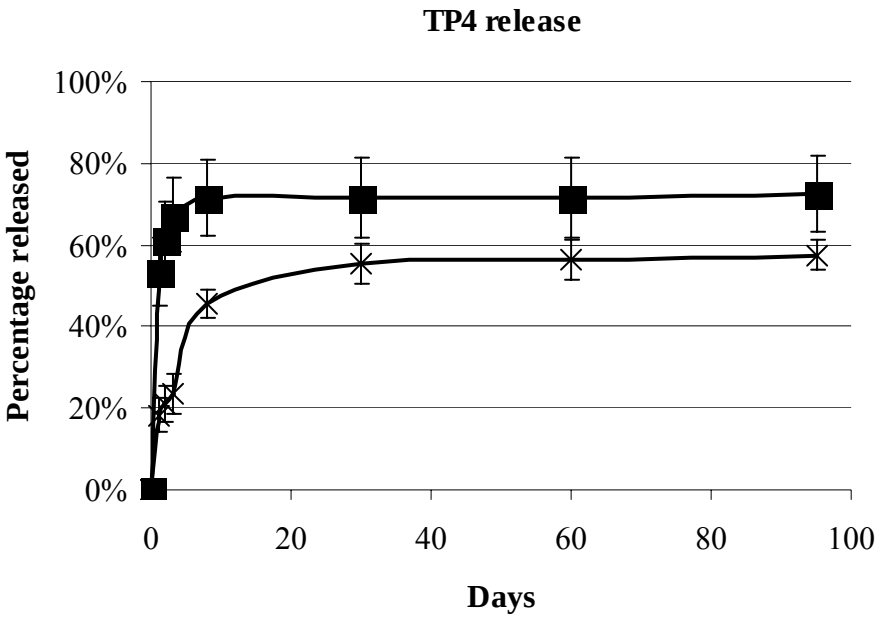

Fig.5: Release kinetics of TP4 from microparticles made from 2% (■) and 20% (×) polymer solutions of PLA

Fig.6:

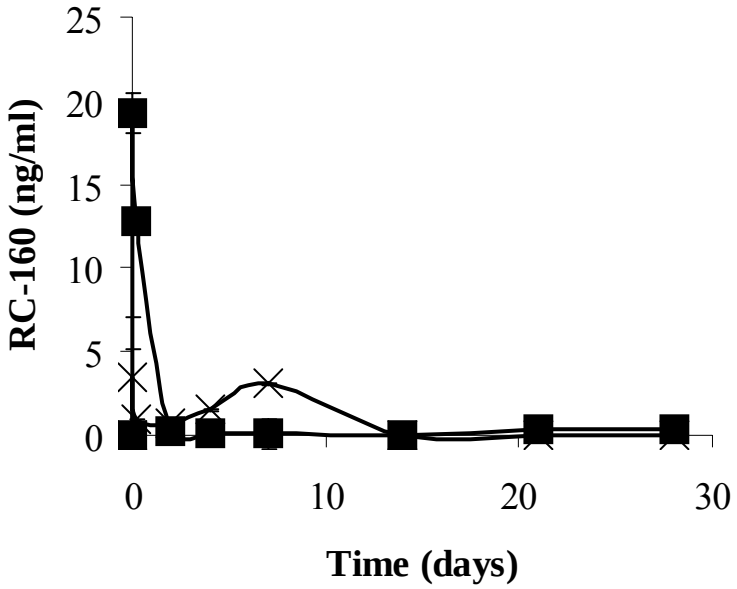

Fig.6: In vivo *release kinetics of vapreotide pamoate from microparticles made from 10% polymer solutions of PLGA 50:50 (■) and 50:50 H (×)*

Tab.1:

Tab.1: Volume weighed mean diameters of different microsphere preparations.

Tab.2:

Tab. 2: Burst releases of different drugs from microspheres made with different polymers within 24 hours.

Drug	Polymer	Polymer concentration (%)	Release after 24 hours (%)
BSA	PLA	2	44.0
		20	32.4
RC-160	PLGA 50:50	10	5.4
	PLGA 50:50H	10	29.7
TP4	PLA	2	53.0
		20	18.0