In vitro and *in vivo* evaluation of a somatostatin analogue released from PLGA microspheres

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Abstract

The purpose of this study was to design poly(lactide-*co*-glycolide) (PLGA) microspheres for the continuous delivery of the somatostatin analogue vapreotide over 2-4 weeks. The microspheres were produced by spray-drying and the desired characteristics, i.e. high encapsulation efficiency and controlled release over 2-4 weeks, achieved through optimizing the type of polymer, processing solvent and co-encapsulated additive. The *in vitro* release was tested in fetal bovine serum preserved with 0.02% of thiomersal. Furthermore, formulations were injected intramuscularly into rats to obtain pharmacokinetic profiles. Encapsulation efficiency was between 34 and 91%, depending on the particular formulation. The initial peptide release (within 6 h) was lowest, i.e. <20%, when acetic acid was used as processing solvent and highest, i.e. 57%, with dichloromethane. The various co-encapsulated additives generally lowered the encapsulation efficiency by 15-30%. The best formulation in terms of low burst and effective drug serum levels (> 1 ng/ml) over 21 to 28 days in rats was the one made with end-group uncapped PLGA 50:50, the solvent acetic acid and the additive polyethyleneglycol. In conclusion, the optimization of formulation parameters allowed us to produce vapreotide-loaded PLGA microspheres of suitable characteristics for therapeutic use.

Keywords: Somatostatin analogue; Microspheres; Release kinetics; PLA/PLGA; Plasma levels

1. Introduction

The medical application of peptides is subject to a number of well-known problems such as limited stability in pharmaceutical formulations or enzymatic degradation and, hence, low bioavailability. Drug delivery systems are generally considered very useful to overcome these difficulties [1]. One way to obtain therapeutic drug levels is by encapsulating peptides into bioerodable polymer microspheres that protect the peptides against inactivation and prolong their release over a few weeks. Furthermore, controlled release parenteral dosage forms may improve the efficacy of drugs by reducing the frequency of injections and by decreasing plasma level fluctuations [2]. Vapreotide, (RC-160, DFCYDWKVCW-NH_2), an analogue of somatostatin, synthesized by Cai et al. [3], has been experimentally shown to be particularly active in various proliferation conditions, e.g., breast and prostate cancers; vapreotide has been successfully used in man, amongst others, against AIDS-related diarrhea [4, 5]. Vapreotide is a typical representative of biologically active peptide drugs with high therapeutic potential, but a relatively short plasma half-life after parenteral administration. Despite the variety of therapeutic activities, vapreotide has only limited clinical usefulness because of the necessity of continuous intravenous infusion. Indeed, a simple parenteral administration of this peptide may not be fully effective, and frequent injections or continuous infusion are required to ensure adequate plasma levels. Therefore, to obtain a long-term and constant therapeutic effect, a sustained release system is needed.

Microparticulate controlled-release systems, prepared from poly(lactide) (PLA) or poly(lactide-co-glycolide) (PLGA), have been widely investigated for the delivery of drugs [6, 7]. PLA/PLGA microparticles appear particularly promising as delivery systems for proteins [8, 9] or peptides [10, 11]. According to previous

studies the release of a molecule from PLG/PLGA microspheres may depend on the polymer (lactic/glycolic acid ratio, molecular weight) [12, 13] and microsphere (size, loading) characteristics [14, 15]. Comparatively little data are available on the effect of co-encapsulated additives on drug release from PLA/PLGA microspheres.

In the present study, several additives were co-encapsulated together with the peptide vapreotide to develop a formulation that delivers this peptide drug in a continuous manner over a period of three weeks to one month. The vapreotide loaded PLA/PLGA microspheres were characterized in terms of size, morphology, *in vitro* peptide release and drug plasma levels in rats.

2. Materials and methods

2.1 Materials

Poly(d,l-lactide) (PLA) and various poly(d,l-lactide-co-glycolide) (PLGA) differing in molecular weight and composition were purchased from Boehringer Ingelheim (Ingelheim, Germany). They included end-group capped and uncapped PLGA 50:50 (Resomer® 502; M_w of approx. 14 kDa), end-group capped PLGA 75:25 (Resomer® 752; M_w of approx. 17 kDa) and PLA (Resomer® R202; M_w of approx. 14kDa). The somatostatin analogue vapreotide pamoate, (DFCYDWKVCW-NH_2), was synthesized by Novabiochem, Laufingen, Switzerland. Chitosan (SEA CURE CL 210) was a gift from Pronova Biopolymer (Lysaker, Norway). Bovine serum albumin (BSA), polyethyleneglycols (PEG 10000, PEG 20000), ethyl stearate, and the analytical grade ethyl formate (EF) and dichloromethane (DCM) were from Fluka (Buchs, Switzerland). Acetic acid was from Merck (Dietikon, Switzerland) and fetal bovine serum from Gibco BRL (Basel, Switzerland).

2.2 Microencapsulation

Vapreotide microspheres were prepared by spray-drying. The peptide was either dissolved in a 5% (w/w) polymer solution in acetic acid or dispersed, by means of magnetic stirring, in a 5% (w/w) polymer solution in DCM or EF. The suspension, containing the polymer and drug, was spray-dried in a laboratory spray-dryer (Mini Spray-Dryer 190, Büchi, CH-Flawil) with the following process parameters: product feed: 3 ml/min; inlet temperature: 50 °C; outlet temperature: 40 °C; aspirator setting: 40 m^3/h; spray-flow: 450 Nl/h. A 0.7 mm nozzle was used throughout the experiments. The following additives were individually co-encapsulated: BSA, PEG 6000, PEG 10000, PEG 20000, chitosan and ethyl stearate. The additives were dissolved or dispersed, by magnetic stirring, in the polymer solution.

The obtained microspheres were further processed following a previously established protocol [8]. Briefly, the particles were washed with a 0.1% (w/w) poloxamer 188 solution and distilled water, and collected on a 0.2 μm cellulose acetate filter. After drying under vacuum (10 mbar) at room temperature for 24 hours, the microspheres were redispersed in hexane to break up any aggregated particles, and dried again under vacuum (10 mbar) for 12 hours. This protocol was found suitable to eliminate efficiently residual amounts of water and organic solvents from the microspheres. Typical ranges of residual organic solvents in the particles were below 200 ppm (unpublished data). The product yield generally lay in the range of 70 to 78% with respect to the amount of starting solid materials.

2.3 HPLC-method for peptide assay

The intact peptide was analyzed 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.

2.4 Morphology, size and calorimetric transitions of microspheres

For morphological examination, the microspheres were mounted on double-face adhesive tape, sputtered with platinum and viewed in a Hitachi S-700 scanning electron microscope (SEM).

Size and size distribution of the microspheres were analyzed by light microscopy (Wild, Heerbrugg, Switzerland) and laser light diffraction (Mastersizer®, Malvern, UK).

Calorimetric transitions of the native drug and microspheres loaded with the drug were determined by differential scanning calorimetry (DSC-7, Perkin Elmer, Hünenberg, Switzerland). The temperature range was from 20 to 230 °C, and the samples heated at a rate of 10 °C/min.

2.5 Determination of vapreotide content in the microspheres

The vapreotide content in the PLGA microspheres was determined by HPLC according to a method described previously [16]. 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 vapreotide was calculated as the ratio of actually measured to theoretical (nominal) drug content in the microspheres.

2.6 NMR study

To verify that vapreotide pamoate was not modified chemically by the exposure to acetic acid during microencapsulation, we have used analytical one- and two-dimensional NMR spectroscopy. During the study 1D proton spectra of

vapreotide pamoate where compared to spectra recorded on vapreotide pamoate that had been pre-treated with acetic acid. For the latter sample, 10 mg of the peptide salt were dissolved in 500 μl of acetic acid, the solvent was evaporated under vacuum (Speed Vac 110, Savant Instruments, Farmingdale, NY, U.S.A.), and the pellet was re-suspended in 500 μl of deuterated dimethylsulfoxide (d_6-DMSO). Furthermore, 10 mg of vapreotide pamoate and 10 mg of vapreotide acetate were separately dissolved in 500 μl of d_6-DMSO. In addition, [^{13}C,^{1}H] correlation experiments were recorded for both the original and the acid pre-treated vapreotide pamoate.

2.7 In vitro *release*

In vitro drug release profiles (up to 28 days) were obtained by incubating the microspheres (approx. 10 mg accurately weighed, n=3) in 4.0 ml of fetal bovine serum preserved with 0.02% (w/w) of thiomersal. Incubation took place in rotating vials at 37 °C. The amount of drug released was determined indirectly by measuring the amount of drug remaining in the microspheres. Indeed, a previous investigation has shown that vapreotide remained intact inside microspheres upon incubation in serum, but degraded substantially, i.e. 85% within 14 days, once released from the microspheres [16]. 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 remaining vapreotide in the microspheres was determined by HPLC, as described above. 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 [16].

2.8 In vivo study

In vivo evaluation of selected formulations (4 types of microspheres) was performed 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% rel. 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 [17] with a sensitivity of 50 pg/ml. Data were presented as mean ± standard deviation. Statistical comparisons were made using Student's t-test, and a P-value lower than 0.05 was considered significant. Correlation between *in vitro* and *in vivo* data was not entirely satisfactory and requires further clarification before it may be presented.

3. Results and discussion

3.1 Morphology, drug content and burst release

The size of the microspheres lay within a relatively narrow range of 1-15 μm. No significant size differences were detectable amongst all the preparations. Representative SEM micrographs of the particles show that the shape of the microspheres was affected by the processing solvent and by the co-encapsulation of additives (Fig. 1). The end-group uncapped PLGA 50:50 microspheres prepared with ethyl formate (Fig. 1A) or acetic acid (Fig. 1C) were spherical and showed no aggregation. In contrast, the microspheres prepared with DCM (Fig. 1B) or with acetic acid and co-encapsulated PEG 20000 (Fig. 1D) had an irregular shape and were aggregated. A similar morphology was seen with end-group capped PLGA

50:50 microspheres containing PEG 20000 (results not shown). These results indicate that both the solvent dichloromethane and the additive PEG 20000 exerted, at least before the final drying of the particles, a plasticizing effect on the microspheres.

Tables 1 and 2 summarize the drug loading and burst release from the microspheres prepared with various polymers, solvents and additives. The highest entrapment efficiency of 91% was achieved with ethyl formate as processing solvent, while only 65-67% efficiency was achieved with dichloromethane and acetic acid (Table 1). This difference of entrapment efficiency cannot, at present, be explained. In contrast to the very satisfactory drug entrapment with ethyl formate, the burst release of drug from the microspheres made with this solvent was rather high, i.e. 44 and 55 % after 1 and 6 h, respectively. In view of a therapeutic use, the microspheres prepared from acetic acid gave a more appropriate burst release, i.e., 11 and 18% after 1 and 6 h, respectively. The higher burst release from the microspheres prepared with ethyl formate or dichoromethane may be explained by the different solubility of the peptide in these solvents. In acetic acid, the peptide had a solubility of > 40 mg/ml, whereas in ethyl formate and dichoromethane the peptide was only slightly soluble (5.3 and 4.5 mg/ml, respectively). For microencapsulation, the peptide had to be suspended in the polymer solutions made with the latter two solvents, whereas it could be dissolved in the acetic acid polymer solution. Thus, the low initial burst release observed when using acetic acid probably indicates a homogenous drug distribution resulting in only minor amounts of peptide located at the particle surface. In any case, the glass transition temperature of the microspheres was comparable between the various preparations and lay between 45 and 50 °C (data not shown). DSC thermograms further suggested formation of a solid solution

between the peptide salt and the PLGA 50:50 microspheres, as endo- and exothermic transitions of the native drug compound (between 130 and 170 °C) were not detectable with the micrsopheres loaded with the drug. Finally, pharmacokinetic experiments in rats revealed a very good agreement between C_{max} and the amount of burst release *in vitro* (data not shown). Therefore, acetic acid was used as processing solvent for all further microsphere formulations.

With the aim to improve the encapsulation efficiency while maintaining the low burst release, various formulation parameters were varied: (i) nominal drug loadings were varied between 5 and 30%; (ii) various additives were co-encapsulated; (iii) end-group capped PLGA 50:50, PLGA 75:25 and PLA were used (Table 2). Increasing the nominal drug loading from 5 to 20% lowered the encapsulation efficiency from 70 to 54%. This is a well described phenomenon [10] that can be related to a higher osmotic pressure of highly loaded matrices and larger pores formed at higher loadings. The addition of additives generally lowered the encapsulation efficiency from 65% (10% nominal loading) to values between 34 (ethyl stearate), 38 (BSA), 40 (chitosan), and 40 to 50% (PEGs) (Table 2). Increasing the nominal amount of PEG 20000 from 10 to 30% also lowered slightly the encapsulation efficiency. The effect of the additives might be ascribed to an increase of porosity of the microspheres. Our rationale for using these additives lay mainly in a potential enhancement of polymeric entanglement (PEG, BSA) and hydrophobicity (ethyl stearate) inside the microspheres and in a ionic interaction (chitosan) with the peptide drug. However, the assumed effects did not occur. Finally, the end-group capped PLGA 50:50, PLGA 75:25 and PLA did not improve the microencapsulation efficiency.

Drug release within the first 6 h (also called burst release) was between 10 and 20% of the actual loading, irrespective of nominal loading (5, 10, 20% in end-group uncapped PLGA 50:50) and polymer type (end-group capped PLGA 75:25, PLA). Similarly, the co-encapsulated additives BSA, chitosan and ethyl stearate had no significant effect on the burst release. By contrast, co-encapsulated PEGs significantly ($P < 0.05$) increased the burst release as compared to the additive-free microspheres. This might be due to the plasticizing effect of this type of additive, as suggested above, or to an increase of hydrophilicity of the microspheres that contain PEGs.

3.2 NMR study

The results from one-dimensional NMR clearly proved that no chemical modification of vapreotide pamoate occurred upon treatment with acetic acid. This is evident from the very high similarity between the 1D spectra of the reference and test samples. However, two signals had different line-shapes in the 1D spectra of the two samples. We suspect this to be due to the more acidic pH in the sample treated with acetic acid. Such a pH-shift alters the exchange rate of labile protons, e.g. hydroxyl or amide protons, with the water that is contained in the sample and hence alters the line-width. We have confirmed this mechanism by supplementing our data with proton, carbon 2D correlation experiments. The experiments gave virtually identical correlation maps for the two forms, a result that is impossible if the sample would have undergone chemical modifications during the acid treatment. Finally, the absence of chemical modifications by acetic acid treatment was also confirmed by mass spectroscopic measurements (results not shown).

3.3 In vitro release kinetics

In a previous study [16], 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 2 depicts the *in vitro* release profiles of vapreotide from microspheres made of end-group uncapped PLGA 50:50 (RG502H) and end-group capped PLGA 75:25 (RG752) and PLA (R202) with nominal drug loadings of 10%. The PLGA microparticles released the peptide considerably faster (88 and 73% in 28 days from PLGA 50:50 and PLGA 75:25, respectively) than the PLA microparticles (24% in 28 days). These observations agree with results published by others [14, 18, 19]. All the profiles showed a modest burst release of 12 - 18%. In contrast to comparable studies, the present release profiles did not exhibit a pronounced triphasic pattern (burst release - dormant period - second release pulse). Further, the minor effect of nominal drug loading (5, 10 and 20%) on the release kinetics is shown in the inset. This suggests a strong interaction between polymer and drug within this loading range. Nonetheless, the slightly increased burst release from the formulation containing nominally 20% drug agrees with results reported by others [20-22].

The co-encapsulation of additives into end-group uncapped PLGA 50:50 microspheres exerted slight effects on the overall release pattern, but not on the duration of release. (Fig. 3). After the burst (discussed above), the release rate was generally lowered when the formulations contained additives. A rather continuous release pattern was observed with the microspheres without additive and those containing PEG 20000. This agrees with release studies of timolol maleate from PLGA 50:50 microspheres containing PEG 6000 [23]. With the additives BSA,

chitosan and ethyl stearate, the profiles became more triphasic, which was most pronounced with the latter two excipients. It has been speculated that the dormant phase of triphasic peptide release profiles might be due to ionic interaction between the anionic polymer chains and peptides, or to collapse of the microporous structure inside the microspheres upon water penetration and polymer swelling [10]. Whether either of these mechanisms play a role in the here presented release remains unknown. Similarly, the discontinuous *in vitro* release of EPO from PLGA 50:50 microspheres was not significantly modified by the various additives used in the study by Morlock et al. [24].

3.4 In vivo study

For the pharmacokinetic study in rats, end-group uncapped PLGA 50:50 microspheres (10% nominal loading, with and without co-encapsulated PEG 20000; 20% nominal loading, no additive) and end-group capped PLA microspheres (10% nominal loading, no additive) were selected (Table 2). Each formulation was injected i.m. into rats, and vapreotide serum concentrations monitored for 28 days (Fig. 4). With the exception of the PLA microspheres, all the other formulations produced drug levels higher than 1 ng/ml for up to at least three weeks. The most prolonged plasma levels appeared with the PLGA 50:50 microspheres with 10% nominal drug loading and co-encapsulated PEG 20000. However, after day 6, the differences in the profiles obtained with all three PLGA 50:50 formulations were statistically not significant ($P > 0.05$). By contrast, PLA microspheres produced detectable serum levels for two days only. These *in vivo* results agree qualitatively quite well with the *in vitro* release profiles (Figs. 2 and 3). Indeed, with the PLGA 50:50 formulations, relatively continuous *in vitro* release was observed over approx. 28 days, whereas with the PLA particles, only very minor amounts of peptide were released after the

initial 6 h (Figs. 2 and 3). Further erosion controlled release was not likely to occur as this homopolymer degrades relatively slowly [8].

Previous experiments with microspheres prepared with dichoromethane or ethyl formate produced very high C_{max}-values (105-135 ng/ml), and plasma levels were maintained over 7-10 days only [16]. Conversely, all microspheres formulations prepared with acetic acid yielded moderate C_{max}-values from 38 to 54 ng/ml one hour after injection (Fig. 4). We may speculate that the very modest burst release *in vitro* and *in vivo* may be ascribed to relatively low amounts of drug located at the surface of the particles. Since acetic acid is a good solvent for both the peptide and the polymer, a homogeneous drug distribution inside the microspheres can be expected.

Another advantage of the processing solvent acetic acid resides in the good safety profile of this solvent. Acetic acid is indeed listed as class 3 solvent in the ICH guidelines [25]. Thus, residual acetic acid in the microspheres, which remains to be determined, should not give rise to safety concerns.

4. Conclusions

The objective of this work was to design degradable microspheres for delivering vapreotide at significant plasma levels over 3-4 weeks. In this work, we: (1) successfully encapsulated vapreotide pamoate in PLA/PLGA microspheres using a spray-drying method, (2) demonstrated that the *in vitro* and *in vivo* release of the peptide depended on the type of polymer, solvent and additive used, and (3) achieved significant drug levels in rats over three weeks after intramuscular administration.

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Figure legends

Figure 1. Scanning electron micrographs of spray-dried end-group uncapped PLGA 50:50 microparticles. Effect of processing solvent and additive: ethyl formate (a); dichloromethane (b); acetic acid (c); acetic acid, PEG 20000 (d). The horizontal dimension of the micrographs corresponds to 46 μm.

Figure 2. *In vitro* release of vapreotide pamoate from PLA and PLGA microspheres in serum, containing 0.02% of thiomersal. Effect of polymer type: end-group uncapped PLGA 50:50 (□); end-group capped PLGA 75:25 (n); end-group capped PLA (s). Inset: Release profiles of uncapped PLGA 50:50 microspheres loaded with nominal 5 (□), 10 (×) and 20% (n) of drug.

Figure 3. *In vitro* release of vapreotide pamoate (10%, w/w, nominal loading) from end-group uncapped PLGA 50:50 microspheres in serum, containing 0.02% of thiomersal. Effect of the presence of co-encapsulated additives (generally 10%, except for ethyl stearate (5%, w/w), with respect to PLGA mass): Chitosan (×), ethyl stearate (◆), PEG 20000 (□) and BSA (■). For comparison, vapreotide release from microspheres without additive is given in Fig. 2.

Figure 4. Plasma levels of vapreotide levels in rats after intramuscular injection of 1.5 mg peptide (base equivalent) contained in various microsphere formulations: end-group uncapped PLGA 50:50, 10% nominal loading, no additive (◆); end-group uncapped PLGA 50:50, 20% nominal loading, no additive (■); end-group uncapped

PLGA 50:50, 10% nominal loading, PEG 20000 (□); end-group capped PLA, 10% nominal loading, no additive (×).

Table 1: Effect of the processing solvent used on the entrapment efficiency (E.E.) and burst release of vapreotide pamoate from end-group uncapped PLGA 50:50 microspheres. The nominal drug loading was 10%, relative to the microsphere mass.

Solvent	E. E. (%)	Peptide released after 1h (%)	Peptide released after 6h (%)
Ethyl formate	91 ± 1	44 ± 1.0	55 ± 0.5
Dichloromethane	67 ± 4	57 ± 0.8	64 ± 10.0
Acetic acid	65 ± 5	11 ± 1.5	18 ± 0.9

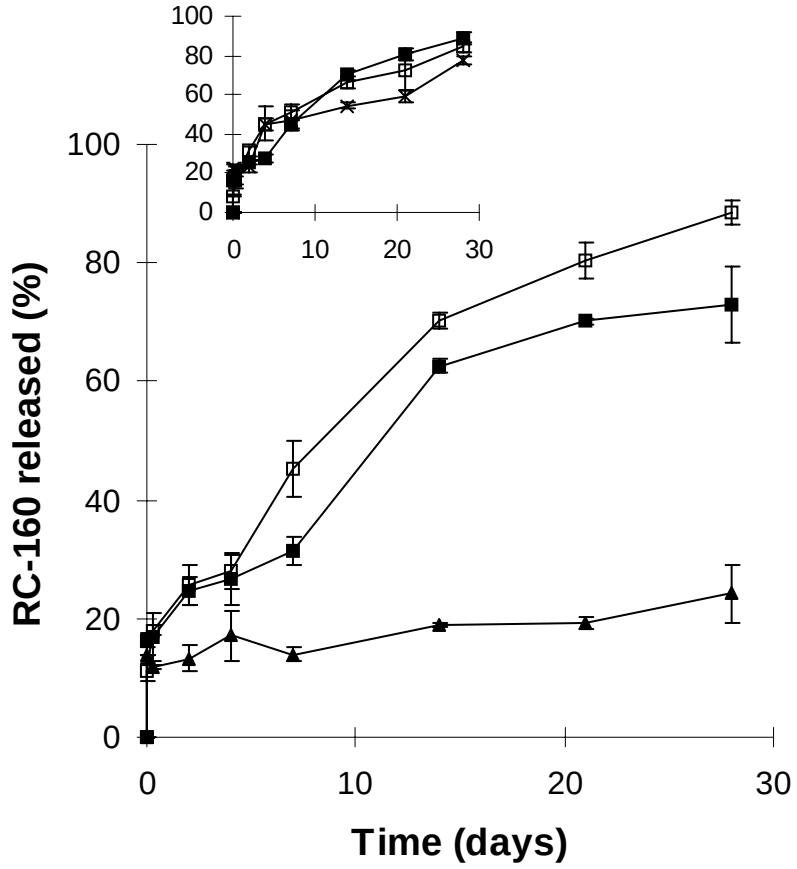
Table 2: Composition, encapsulation efficiency (E.E.) and burst release (within 6 h) of biodegradable PLA and PLGA microspheres containing vapreotide pamoate. The processing solvent was acetic acid.

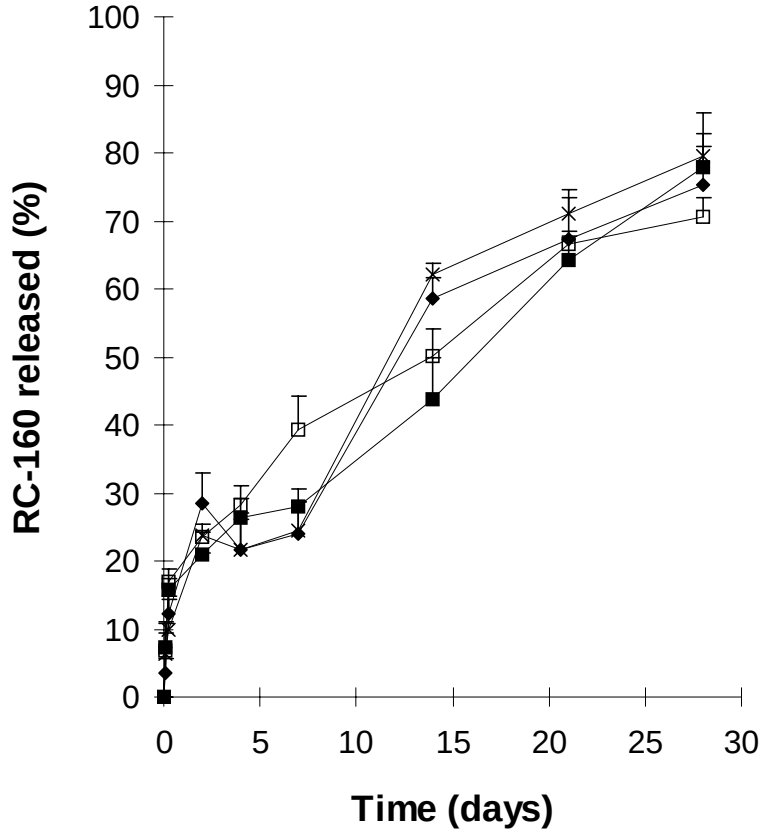
Polymer type[1]	Nominal drug loading (%)	Additive[2]	E. E. (%)	Peptide released after 6 h (%)
502H	5	-	70 ± 2	16 ± 2
502H[3]	10	-	65 ± 5	22 ± 1
502H[3]	20	-	53 ± 2	17 ± 4
502H	10	PEG 10000	40 ± 2	33 ± 5
502H[3]	10	PEG 20000	50 ± 3	32 ± 10
502H	20	PEG 20000	40 ± 2	30 ± 2
502H	30	PEG 20000	38 ± 2	30 ± 2
502H	10	BSA	38 ± 6	16 ± 2
502H	10	Chitosan	40 ± 5	8 ± 4
502H	10	Ethyl stearate	34 ± 5	12 ± 2
502	10	PEG 20000	33 ± 3	29 ± 5
752	10	-	75 ± 9	17 ± 4
202[3]	10	-	73 ± 10	11 ± 3

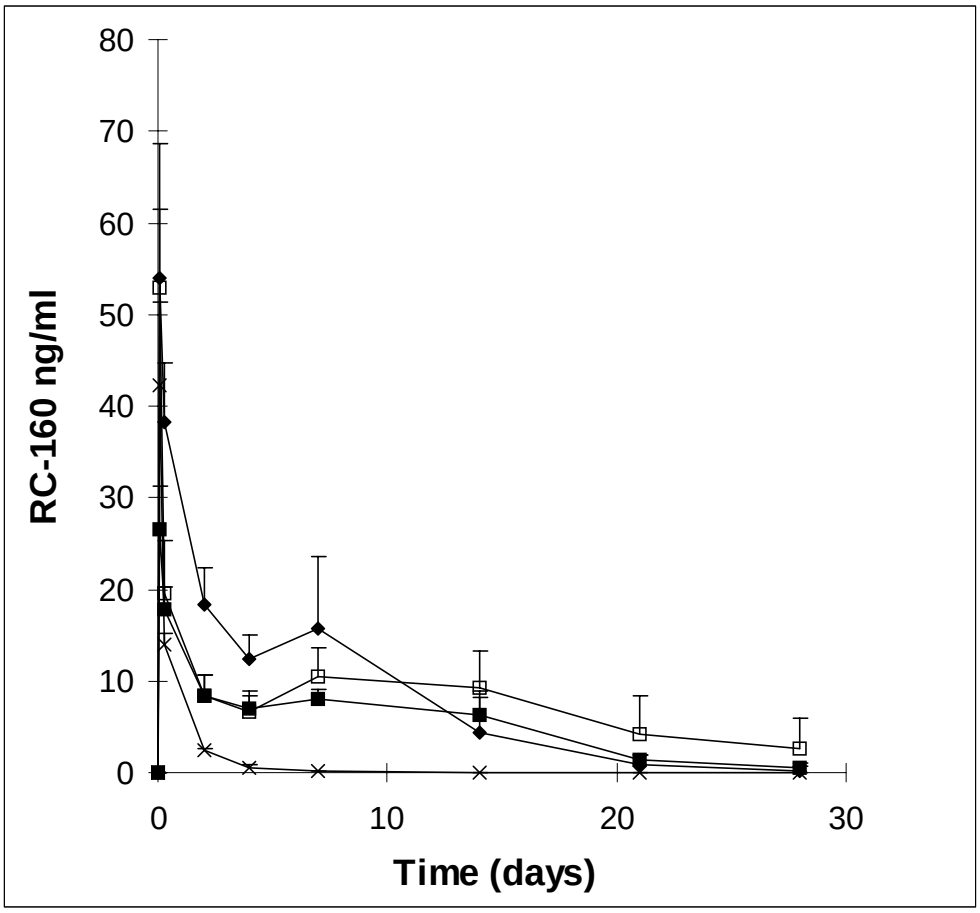
[1] 502H, 502, 752 and 202 refer to end-group uncapped and capped PLGA 50:50, end-group capped PLGA 75:25 and PLA, respectively.

[2] The nominal amount of co-encapsulated additive was generally 10%, relative to the PLA/PLGA-mass, except for ethyl stearate (5%).

[3] Formulations selected for *in vivo* study.


RC-160 released (%)
Time (days)
0
20
40
60
80
100
0
10
20
30


RC-160 released (%)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (days)


80
70
60
50
40
30
20
10
0
RC-160 ng/ml
0
10
20
30
Time (days)