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Importance of the test medium for the release kinetics of a somatostatin analogue from poly(D,L-lactide-*co*-glycolide) microspheres

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Abstract

The determination of in vitro release kinetics of peptides from poly(D,L-lactide-*co*-glycolide) (PLGA) microspheres generally requires optimization of the test conditions for a given formulation. This is particularly important when in vitro/in vivo correlation should be determined. Here, the somatostatin analogue vapreotide pamoate, an octapeptide, was microencapsulated into PLGA 50:50 by spray-drying. The solubility of this peptide and its in vitro release kinetics from the microspheres were studied in various test media. The solubility of vapreotide pamoate was approximately 20–40 μg/ml in 67 mM phosphate buffer saline (PBS) at pH 7.4, but increased to approximately 500–1000 μg/ml at a pH of 3.5. At low pH, the solubility increased with the buffer concentration (1–66 mM). Very importantly, proteins (aqueous bovine serum albumin (BSA) solution or human serum) appeared to solubilize the peptide pamoate, resulting in solubilities ranging from 900 to 6100 μg/ml. The release rate was also greatly affected by the medium composition. Typically, in PBS of pH 7.4, only $33 \pm 1\%$ of the peptide were released within 4 days, whereas 53 ± 2 and $61 \pm 0.9\%$ were released in 1% BSA solution and serum, respectively. The type of medium was found critical for the estimation of the in vivo release. The in vivo release kinetics of vapreotide pamoate from PLGA microspheres following administration to rats were qualitatively in good agreement with those obtained in vitro using serum as release medium. Finally, sterilization by γ-irradiation had only a minor effect on the in vivo pharmacokinetics.

Keywords: Somatostatin analogue; Microencapsulation; Solubility; Release kinetics; Microspheres; Poly(D,L-lactide-*co*-glycolide); Stability

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1. Introduction

Peptide drugs are excellent candidates for microencapsulation into biodegradable injectable microspheres made of poly(D,L-lactide) (PLA) or poly(D,L-lactide-*co*-glycolide) (PLGA). Entrapment in PLA/PLGA microspheres can protect the peptide from proteolysis and prolong its release and bioavailability. Various peptide drugs have been encapsulated in this type of delivery system including thyrotropin releasing hormone (Hashimoto et al., 1993; Miyamoto et al., 1993), several LH–RH analogues (Ogawa et al., 1989; Okada et al., 1988, 1989; Stoeckemann and Sandow, 1993), octreotide (Bodmer et al., 1992) or cholecystokinin agonist (Blanco-Príeto et al., 1996, 1997).

Most of the available reports describe highly water soluble forms of peptides, such as base and acetate. Depending on the drug, polymer type and test conditions, the in vitro peptide drug release from PLA/PLGA microspheres is generally prolonged over a few weeks and follows either a continuous or pulsatile pattern (Bodmer et al., 1992; Thomasin et al., 1996). While the effect of the polymer characteristics, e.g. molecular weight, and copolymer composition on the prolonged release has been quite well investigated (Heya et al., 1991; Kissel et al., 1991; Bodmer et al., 1992), the importance of the in vitro release conditions seems to be less well defined. Generally, microspheres are incubated in a certain volume of release medium consisting of phosphate buffer saline (PBS), a preservative and, optionally, a surfactant. The test is then performed in glass or plastic tubes or flasks at 37°C under some agitation. Alternative experimental set-ups have been studied for a non-peptide drug and shown to affect the release kinetics for a given formulation (Conti et al., 1995).

For peptide and protein drugs, the composition of the release medium is most important (Bodmer et al., 1992; Park et al., 1995; Yang and Cleland, 1997; Johansen et al., 1998). For octreotide, the release slowed down with increasing ionic strength (Bodmer et al., 1992). For BSA, 20% of the dose was released after a few days in phosphate buffer saline, whereas 80% of the dose was released after 6 h in small intestinal fluid (Russel-Jones and Jeffery, 1994). Another issue is the formation of acidic polymer degradation products leading to a pH change of the medium during release studies (Park et al., 1995). Continuous removal of these acidic moieties by dialysis would be desirable, but is technically cumbersome (Park et al., 1995). On the other hand, the commonly used technique of replacing the release medium at the sampling time points or upon pH drop appears rather accidental. Thus, a practical and well validated method of maintaining the pH does not seem to be available.

Besides the release kinetics, the stability of the released material in the medium is also most important. Most peptides and proteins are not stable in buffer media at 37°C. Chemical degradation (cleavage, oxidation, reduction, etc.) and physical changes (conformational, aggregation, adsorption on surfaces) have been reported (Park et al., 1995; Morlock et al., 1997; Johansen et al., 1998). Considering all these aspects, it becomes clear that in vitro release tests are complex experiments which require careful attention for each individual formulation. This may be even more critical for peptide salts of poor water solubility, such as peptide pamoate or tannate.

The goal of the present work was to determine an appropriate in vitro release test medium for microencapsulated vapreotide pamoate, a poorly water soluble somatostatin analogue. The pharmacological importance of this drug has been recently summarized by Rothen-Weinhold et al. (1997). Therapeutically, it is of interest for the treatment of, among others, acromegaly and neuroendocrine tumours. Irradiated and non-irradiated microparticles were injected intramuscularly in rats, and the plasma levels were assessed.

2. Materials and methods

2.1. Materials

PLGA (50:50) with free carboxyl end groups was purchased from Boehringer Ingelheim (Ingelheim, Germany; RG 502H, inherent viscosity of 0.2 dl/g). The somatostatin analogue vapreotide pamoate (D-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH_2) was synthesized by Novabiochem, Laufin-

gen, Switzerland. Bovine serum albumin (BSA) and the organic analytical grade solvents ethyl formate (EF), dichloromethane (DCM), nitromethane, nitroethane, *n*-propanol and dimethylsulfoxide (DMSO) were from Fluka (Buchs, Switzerland). Foetal bovine serum was obtained from Gibco BRL (Basel, Switzerland) and human serum albumin (HAS) (Albumin 20% SRK) was from the ZLB Zentrallaboratorium (Bern, Switzerland).

2.2. Peptide solubility

The solubility of the vapreotide pamoate was studied in different media: (i) phosphate buffer saline (PBS) of different pH and molarities, and with or without 1, 5 and 10% BSA or 1, 4 and 10% HSA; (ii) in serum (S), serum ultrafiltrates (SU) (cut-off 5000 and 100 000 Da), and mixtures thereof at S:SU ratios of 100:0, 10:90, 1:99 and 0:100. We used serum ultrafiltrate to mimic the interstitial environment. The solubility was determined by incubating an excess of peptide in 4 ml of test medium at room temperature for 8, 24 and 48 h. The dispersions were magnetically stirred. The samples were centrifuged for 10 min at $10\,000 \times g$ and the solubilized peptide was assayed by high performance liquid chromatography (HPLC), as specified later.

2.3. Peptide stability

Peptide stability was determined in 67 mM PBS of pH 7.4 and in serum upon incubation at 37°C. Periodically (1, 3, 5, 7, 10 and 14 days), aliquots were taken and the amount of intact peptide was assayed by HPLC using the procedure described next.

2.4. HPLC method for peptide assay

The intact peptide was analyzed by HPLC (Column Licrospher® RP-18, 4 × 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.

2.5. Microencapsulation

The peptide was either dispersed in a 5% (w/w) polymer solution in DCM or EF, or dissolved in the solvent mixtures described in Table 2. The suspension or solution containing the polymer and drug was spray-dried (nozzle of 0.7 mm diameter) in a laboratory spray-dryer (Mini Spray-Dryer 190, Büchi, CH-Flawil) at a rate of 3 ml/min. The process was run under a flow of pressurized air of 450 Nl/h, a flow of drying air of 40 m^3/h, and inlet and outlet temperatures of 50 and 40°C, respectively. The obtained microspheres 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 at room temperature for 24 h, the microspheres were redispersed in hexane to break up any aggregated particles, and dried again under vacuum for 12 h. A fraction of the preparation was sterilized by γ-irradiation (^{60}Co source, 0.797 kGy/h) with 25 kGy.

2.6. Microsphere characterization

The morphology and size of the microspheres were analyzed by light microscopy (Wild, Heerbrugg, Switzerland) and laser light diffraction (Mastersizer®, Malvern, UK). The drug content in the microparticles was determined by first dissolving 10 mg of microspheres in 3 ml of acetonitrile, then 2 ml of chloroform were added to the suspension and the peptide extracted three times with 2 ml of triethylamino phosphate buffer of pH 2.3. The peptide was assayed in the aqueous phase by HPLC, as already described.

2.7. In vitro release

The in vitro drug release was determined by dispersing a precisely weighed amount of microspheres in 4.0 ml of either iso-osmolar PBS (pH 7.4, 67 mM phosphate), aqueous solutions of 1% (w/w) BSA, or serum. All test media were preserved with 0.02% (w/w) of thiomersal. Incubation took place in rotating vials at 37°C. At regular intervals, the samples were centrifuged, and the amount of peptide remaining in the mi-

crospheres was determined by HPLC, as previously specified. The HPLC assay detected the intact, unreleased peptide.

2.8. In vivo study

One particular microsphere preparation (the formulation prepared with ethyl formate alone, wherein the peptide was suspended) was tested in rats (male Sprague–Dawley rats, 380–400 g; C.C.R.J., Les Genest St. Isle, France). Rats were housed in groups of four in a well-ventilated environment under controlled temperature (22 ± 1°C) and humidity (50 ± 5%), with food and water made available ad libitum. The animals were taken care of in accordance with the UFR des Sciences Pharmaceutiques et Biologiques Local Ethical Committee and the NIH Guidelines for the Care and Use of Laboratory Animals (1985).

Twelve animals were divided into two groups. One group received intramuscularly γ-radiation sterilized microspheres, and the other group non-sterilized microspheres. The dose of peptide per rat was 1.5 mg (amount calculated as vapreotide base). At different time intervals, 1.5 ml (0, 1 and 6 h) or 2.5 ml (2, 4, 7, 14, 21 and 28 days) of blood were collected. Vapreotide plasma levels were assessed by radioimmunoassay (Manson-Garcia et al., 1988).

3. Results and discussion

3.1. Peptide solubility

The solubility of vapreotide pamoate in PBS increased with time (8, 24, 48 h) and reached an apparent equilibrium after 48 h. This time dependency was particularly pronounced at low pH (3.5 versus 5.5, and 7.4). At the lower pH values, the vapreotide pamoate was substantially more soluble (Fig. 1), which must be ascribed to an increased dissociation of the peptide salt. At pH 3.5, the solubility also increased with the molarity of the medium (Fig. 1). The solubility of vapreotide pamoate was greatly enhanced in protein containing media such as aqueous BSA solutions and serum, whereas in serum ultrafiltrates, it was comparable with that in PBS at pH 7.4 (Table 1, Fig. 1). In the protein containing media, maximum solubility was observed already after 8 or 24 h, whereupon the amount of peptide in solution decreased gradually. Thus, in the protein containing media, no equilibrium was observed. This decrease in peptide concentration over time was due to peptide degradation, which was significant after 8 h (serum) or 48 h (BSA medium) already (see later). The data also reveal that the vapreotide solubility depended on the protein concentration in the test solutions, with the solubility increasing from approximately 900 to 6100 mg/ml when the BSA concentration was lifted from 1 to 10%. Similar results were obtained with HSA (data not shown). These findings may be partly explained by the binding in vitro of the peptide to plasma proteins. Typically, vapreotide binds 80% to albumin (mole% of bound peptide to total peptide) and 1% to α-glycoproteins. Furthermore, we assume that albumin has solubilized the peptide. Such a solubilizing effect of albumin has already been reported for an aldose reductase inhibitor, for which the solubility increased about 17- to 57-fold in the presence of 6×10^{-4} M (approximately 4%, w/w) HSA, as compared with buffer solutions or pure water (Kurono et al., 1987).

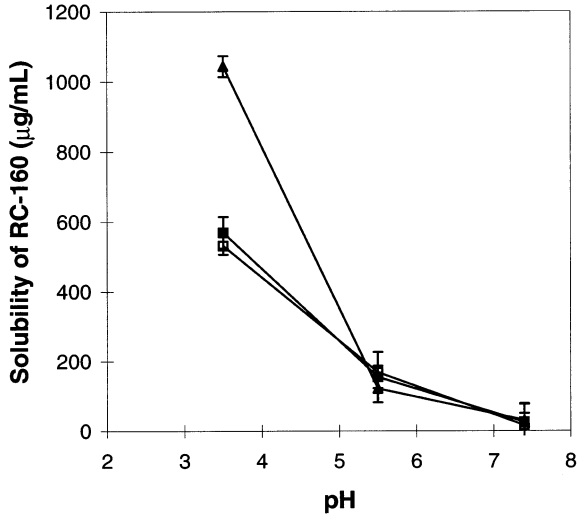

Fig. 1. Solubility of vapreotide pamoate in PBS at various pH and molarity values: 1 mM (□), 10 mM (■), 66 mM (▲) ($t = 24$ h).

Table 1
Solubility of RC-160 in serum (S), serum ultrafiltrates (SU), and aqueous BSA solutions ($n = 3$)

Medium	Time (h)	Solubility (μg/ml)
Serum	8	920 ± 45
	24	745 ± 37
	48	594 ± 20
Serum ultrafiltrate[a]	8	42 ± 5
	24	30 ± 3
	48	27 ± 4
S:SU (10:90)	8	103 ± 11
	24	82 ± 10
	48	71 ± 10
S:SU (1:99)	8	32 ± 3
	24	28 ± 2
	48	24 ± 3
BSA, 1%	8	394 ± 59
	24	903 ± 40
	48	520 ± 39
BSA, 5%	8	2736 ± 59
	24	3131 ± 53
	48	2698 ± 88
BSA, 10%	8	5524 ± 330
	24	6128 ± 450
	48	3185 ± 172

[a] Cut-off of the membrane was 5 kDa; a 100 kDa cut-off membrane was also used, but the resulting peptide solubility in the ultrafiltrate was not significantly different.

3.2. Peptide degradation

In vitro degradation of vapreotide pamoate at 37°C over prolonged periods of time was quite important in both PBS (31% degraded within 14 days) and serum (85% degraded within 14 days) (Fig. 2). The increased degradation in the protein containing media can be attributed to the higher solubility of the peptide in these media. If the degradation kinetics was fitted to a first-order reaction, the rate constants were 2.52×10^{-2} per day ($r^2 = 0.971$) in PBS and 12.87×10^{-2} per day ($r^2 = 0.959$) in serum. Fits were exclusively for the purpose of determining an order of magnitude of the degradation rates. As various unidentified degradation steps were expected to occur, the first-order model may not be adequate. Particularly in serum, a second-order model was actually better ($k = 4.01 \times 10^{-3}$ ml/mg/day; $r^2 = 0.981$). The occurrence of various degradation steps and products was revealed by HPLC chromatograms. The retention time of intact vapreotide was 17.7 min. In PBS, the first degradation products appeared on day 3 of incubation with retention times of 14.3 and 17.1 min. On day 5 of incubation, two additional peaks appeared with retention times of 9.4 and 20.9 min. No attempts were made here to identify these degradation products. On the other hand, degradation products were not distinguishable in serum, because of the great number of ghost peaks from the serum itself.

3.3. Microsphere characteristics

The microspheres had a regular spherical morphology. The particle sizes had a unimodal distribution with diameters between 1 and 12 μm, and a mean diameter of 3.5 μm. Several microsphere formulations were prepared from different solvents for the peptide and polymer (Table 2). The solvents were chosen on the basis of thermodynamic interaction data (Blanco-Príeto et al., 1998). When the peptide powder was suspended in the polymer solution rather than dissolved in a second solvent system, the encapsulation efficiencies were 67 and 99% with dichloromethane and ethyl formate being used as polymer solvents, respectively. This result might be ascribed to a

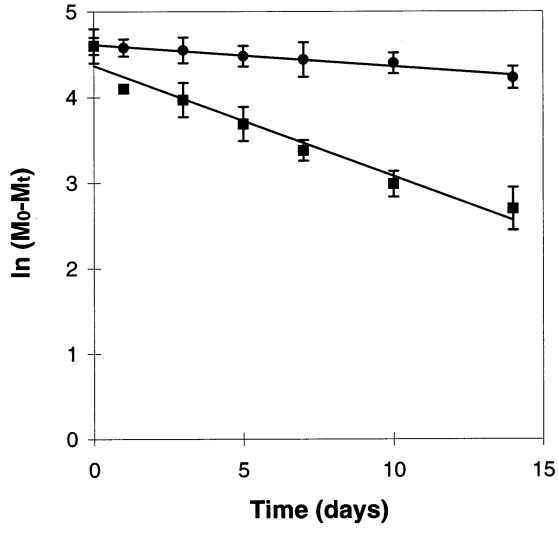

Fig. 2. First-order degradation kinetics of vapreotide pamoate in serum (■) and PBS (●), at 37°C.

Table 2
Encapsulation efficiency (EE) of vapreotide pamoate in PLGA microspheres using different solvent systems ($n = 3$)

Solvent for the drug	Solvent for the polymer	EE (%)
–[a]	Dichloromethane (DCM)	67 ± 4
–[a]	Ethyl formate (EF)	99 ± 1
Aqueous (pH 3.5)	EF	80 ± 6
n-Propanol–water	DCM	93 ± 5
n-Propanol–water	EF	88 ± 2
n-Propanol–water	DCM–nitromethane[b]	81 ± 3
n-Propanol–water	DCM–nitroethane[b]	98 ± 1
n-Propanol–water	DCM–nitroethane[b]	86 ± 4
DMSO–water	DCM–nitroethane[b]	85 ± 3
DMSO–water	DCM–nitroethane[b]	98 ± 3

[a] The peptide powder was dispersed in the polymer solution by ultrasonication and the suspension was stirred during spray-drying.
[b] Molar ratio, 7:3.

higher affinity of the peptide salt to dichloromethane as compared with ethyl formate. Due to the possible higher affinity, some drug is transported to the particle surface upon solvent evaporation during spray-drying. This surface located drug was then eliminated during the washing process of the powder. When the peptide was dissolved in a acidic aqueous solution (pH 3.5) and this solution dispersed in a polymer solution made with ethyl formate, the encapsulation efficiency was significantly lower (80%) than when the drug powder was suspended in a corresponding polymer solution (99%). Furthermore, when propanol–water (molar fraction of 0.2) or dimethylsulfoxide–water (molar fraction of 0.4) mixtures were used to solubilize the peptide, highest encapsulation efficiencies (98%) were observed with the dichloromethane–nitroethane mixture (molar ratio of 7:3) as polymer solvent. Significantly lower encapsulation was generally attained (81–93%), when either dichloromethane, ethyl formate or the mixture dichloromethane–nitromethane were used as polymer solvents. Generally, these differences may be explained by the low polymer–solvent interaction energy of the dichloromethane–nitroethane system, as described recently (Blanco-Príeto et al., 1998). The formulation prepared with the solvent ethyl formate alone, wherein the peptide was suspended, was used for all further experiments.

3.4. In vitro release kinetics

The in vitro release of vapreotide pamoate from the microspheres depended greatly on the type of release medium (Fig. 3). In PBS of pH 7.4, only 33 ± 1% of the peptide were released within 4 days, whereas 53 ± 2 and 61 ± 0.9% were released in 1% BSA solution and serum, respectively (Fig. 3). The faster release in the latter two media may be ascribed to the much higher solubility of the peptide in these media (Table 1). In serum, a total of 67 ± 0.7% was released within 8 days. In PBS and 1% BSA solution, a total amount of 46 ± 1 and 74 ± 1.2%, respectively, were released after 8 days. These continuous release profiles contrast with the often observed pulsatile pattern reported for peptide drugs (Sanders et al., 1984; Lawter et al., 1987; Bodmer et al., 1992).

3.5. In vivo study

After intramuscular injection of γ-irradiated and non-irradiated microspheres in rats (the formulation prepared with the solvent ethyl formate

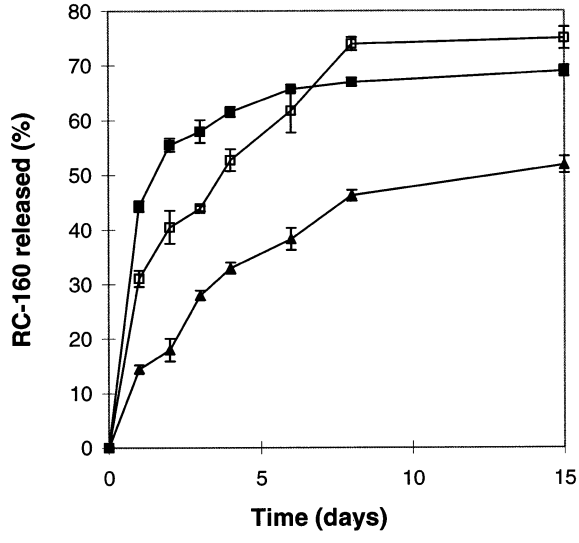

Fig. 3. In vitro release kinetics of vapreotide pamoate from PLGA microspheres in BSA 1% (□), serum (■) and PBS (▲).

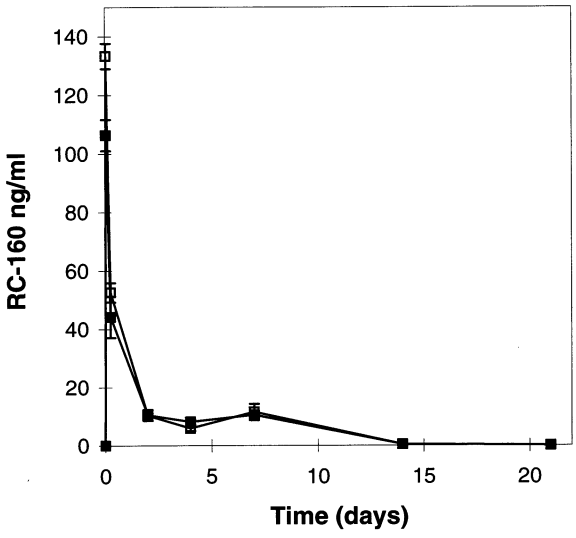

Fig. 4. In vivo release kinetics profiles of RC-160 following i.m. administration of γ-irradiated microspheres (□) or non-irradiated microspheres (■) in rats (1.5 mg equivalent peptide).

alone wherein the peptide was suspended), the vapreotide plasma levels showed an initial peak followed by a plateau at approximately 10 ng/ml over 1 week (Fig. 4). After 1 week, the plasma level dropped off and reached undetectable levels after a second week. Although the maintenance of therapeutic plasma levels (1 ng/ml) over approximately 12 days may have clinical importance, clinical testing of the present formulation cannot be envisaged because of the high burst release, potentially including toxic effects. Sterilization by γ-irradiation had only a minor effect on the pharmacokinetic profiles. Further, the in vitro release of the peptide from irradiated and non-irradiated microspheres showed no significant difference (data not shown); this is in good agreement with the marginal (approximately 5%) molecular weight loss of the polymer used after γ-sterilization (Rothen-Weinhold et al., 1997). These in vivo results were qualitatively in good agreement with those of the in vitro release in serum. Data from in vitro release studies may not always give an insight into the performance of a dosage form in vivo. However, selection of appropriate release test media and conditions should provide meaningful in vitro release data for further development and improvements of dosage form design. Gido et al. (1994) have found a better correlation using diluted plasma as dissolution medium as compared with phosphate buffer (pH 7.4) for microspheres embedding doxepin. With thyrotropin releasing hormone, a relatively good agreement was found between in vitro and in vivo release from PLGA 75:25, when the release medium was a 0.033 M citrate–phosphate buffer at pH 7 (Heya et al., 1994). In the present case, vapreotide pamoate may diffuse rapidly through the endothelium of capillaries and enter the systemic circulation very quickly. Therefore, we used serum as release medium to simulate a physiological environment. The improved agreement between in vitro and in vivo results, when using serum as release medium, may be due to the high solubility of vapreotide in serum and binding to plasma proteins. For vapreotide, one may conclude that the proper choice of the in vitro release test medium is very important for the estimation of in vivo release. This aspect has to be tested with every drug encapsulated in microspheres.

4. Conclusion

The peptide vapreotide pamoate was very efficiently microencapsulated (up to 99%) into end-group uncapped PLGA 50:50 by spray-drying. The differences in release profiles observed in different media underline the importance of testing the in vitro release in an appropriate fluid simulating in vivo conditions. Further experiments are under way to reduce the burst and extend the release of RC-160 from microparticles over 1 month.

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