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Research papers

Preparation and evaluation of insulin-loaded poly(DL-lactide) microspheres using an experimental design

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Abstract

Twenty formulations of bovine insulin in poly(DL-lactide) (DL-PLA) microspheres were elaborated to study the influence of polymer molecular weight, insulin content and polyvinylalcohol (PVA) concentration on microsphere characteristics and in vitro release profile. Ten of them released nearly all the incorporated insulin in 24 h. Of the rest, four were porous, presenting a burst effect over 40% and releasing 70% in 48 h. The other six formulations were non-porous; four released the insulin in 1 week and the other two only released 28% in 15 days. None of these 10 formulations were able to release the total amount of incorporated insulin, probably due to the adsorption of insulin by the polymer. From the results obtained with the experimental design, one more lot was prepared and tested in vitro and in vivo. The in vitro release profile was much slower than the in vivo one; 16% and 28% were released in vitro on the first day and in the following 10 days, respectively, while the corresponding figures for in vivo release were 60% and 90%.

Keywords: Insulin; Poly(DL-lactide); Microspheres; Prolonged release; In vitro–in vivo comparison

1. Introduction

Most peptides and proteins are not well absorbed by the oral route since they lose potency in the digestive tract and they must be administered parenterally. The treatments for which these therapeutic agents are prescribed require stable levels over prolonged periods, which has led to the development of sustained release systems.

In recent years, interest has focused on the use of microspheres prepared from polyesters such as poly(DL-lactide-co-glycolide) (DL-PLGA) and

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poly(DL-lactide) (DL-PLA) as controlled release protein/peptide delivery systems. DL-PLA and DL-PLGA microspheres are commonly prepared by oil-in-water (o/w) organic solvent evaporation (Hora et al., 1990; Jalil and Nixon, 1990). However, this technique has poor encapsulation efficiency of the water-soluble core materials. Many peptides and proteins are water-soluble and poorly soluble in organic solvents. Encapsulation efficiency of these water-soluble compounds within DL-PLGA and DL-PLA microspheres has been improved by using organic phase separation (Ruiz et al., 1990; McGee et al., 1995) and more commonly a water-in-oil-in-water (w/o/w) solvent evaporation technique, developed by Ogawa et al. (1988), which was adopted for microencapsulation water-soluble drugs (O'Hagan et al., 1994; Yan et al., 1994) and vaccines (Alonso et al., 1993; Blanco et al., 1994).

In this paper, DL-PLA microspheres with different molecular weights, insulin content and polyvinylalcohol (PVA) concentration in the external aqueous phase were elaborated in order to evaluate the influence of these three variables on the characteristics of microspheres and on polypeptide release profile, using a central composite rotable design. Insulin was chosen as a polypeptide model drug.

2. Materials and methods

DL-PLA of different molecular weights was performed by ring-opening polymerization of DL-lactide, using tetraphenyl tin (Merck) as catalyst, described by Kulkarni et al. (1966). The experimental synthesis conditions, time, temperature, and catalyst concentration were set up according to Munguia et al. (1992). Molecular weights were determined by gel permeation chromatography (GPC) using a Waters® chromatograph. Four columns of different pore size (Ultrastyragel®) in a row were used with tetrahydrofuran (Merck) as solvent. To calibrate the system, polystyrene monodisperse standards (Tokyo Soda) of various molecular weights were used. Polyvinylalcohol (PVA) of average molecular weight 30 000–70 000 and bovine pancreas insulin (BI) were purchased from Sigma. All the other chemicals were of reagent grade.

Table 1
Coded levels corresponding to the central composite rotational design for polymer molecular weight (X_1), initial percentage of insulin (X_2) and PVA concentration in the external aqueous phase (X_3)

Level	X_1	X_2	X_3
−1.682	40 000	3	0.004
−1	60 000	8.5	0.01
0	90 000	16.5	0.13
1	120 000	24.5	1.6
1.682	140 000	30	4

2.1. Experimental design

The following quadratic model was used,

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{\substack{i=1,j=1 \\ i \neq j}}^{3} b_{ij} X_i X_j + \epsilon$$

where $i = 1, 2, 3$ for the variables average molecular weight of polymer (M_w), theoretical content of insulin and PVA concentration in the external aqueous phase, respectively. Y denotes the response and ϵ is the error, which is assumed to be $N(0, \sigma^2)$.

A central composite rotable design was used which contained 14 different combinations of the variables and the central point was replicated six times. Table 1 shows the coded levels and values of design variables.

2.2. Microspheres preparation

DL-PLA microspheres were prepared by a double emulsion method which is a modification of that described by Ogawa et al. (1988). Briefly, BI was dissolved in aqueous 30% acetic acid (Merck) and poured into a solution of DL-PLA in dichloromethane (Merck). The mixture was sonicated (Sonifier B-12) at output 4 (50 W) for 1 min to form the first inner emulsion (w/o). The BI concentration was kept constant at 7×10^{-2} mg/μl and DL-PLA concentrations in the organic phase were 50–119 mg/ml, the phases ratio (H_2O/

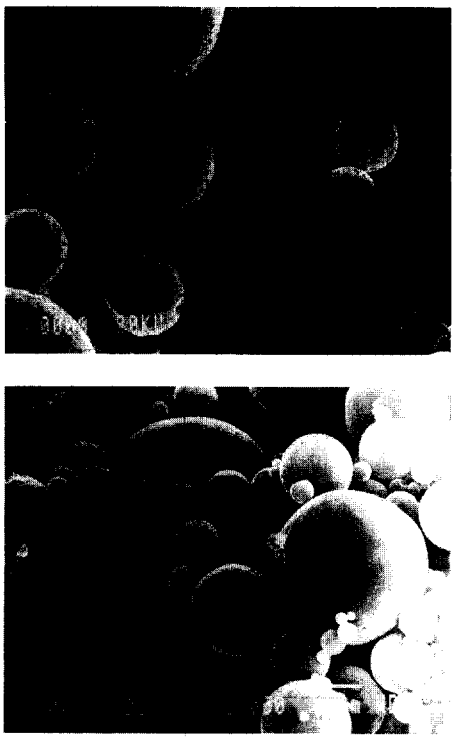

Fig. 1. Scanning electron micrographs of insulin-loaded DL-PLA microspheres prepared by double-emulsion technique: (a) lot 4 porous microspheres; (b) lot 13 (non-porous microspheres). Bar represents 10 μm.

$MeCl_2$) ranging from 1:2.6 to 1:30. The first emulsion was poured into 400 ml of an aqueous solution of PVA under vigorous mixing using a homogenizer (Ultra-Turrax T25; Janke and Kunkel IKA-Work) at 8000 rpm at 5°C for 10 min to form the second emulsion (w/o)/w. The resulting double emulsion was continuously stirred for 3 h at 250 rpm at room temperature until most of the organic solvent evaporated. The microspheres were collected by filtration through an aqueous Millipore® filter (0.45 μm) and dried in a vacuum for 24 h.

2.3. Determination of the BI content in microspheres

BI in the microspheres was determined by a high performance liquid chromatography (HPLC) procedure using a Waters equipment with an ultraviolet (UV) detector under the following conditions: column, Deltapack-C_{18} at room temperature; mobile phase, a mixture consisting of 74 volumes of 0.2 M sodium sulphate anhydrons (Merck), adjusted with phosphoric acid (Merck) to pH 2.3, and 26 volumes of acetonitrile (Merck); flow rate, 1 ml/min; wavelength, 214 nm. The retention time of BI was 7.55 min.

The microspheres (20 mg) were dissolved in 2 ml 90% acetonitrile aqueous solution and BI was extracted into 5 ml 0.05 N hydrochloric acid. The aqueous solution was filtered through a 0.45-μm filter (Millipore® HA). BI in the aqueous solution was assayed by HPLC.

2.4. Determination of microparticle size

The particles were dispersed in 0.1% Tween®-80 (Merck) solution and sized by laser diffractometry using a Coulter counter (Coulter® Multisizer II) after treatment in a bath sonication for 3 min to bring about disaggregation. Particle sizes are expressed as volume mean diameters (vmd) in μm.

2.5. Surface morphology

The shape and surface characteristics of the dried microspheres were examined by scanning electron microscopy (SEM, Hitachi S-450). Samples for SEM were mounted on metal stubs and coated with gold/palladium to a thickness of 200–500 Å under argon atmosphere.

2.6. In vitro release study

Microspheres were suspended in a volume of the medium to obtain 80–90 μg/ml as the final concentration of BI released. The release medium was 0.066 M isotonic phosphate buffered saline (PBS) (pH 7.4) containing 0.02% sodium azide (Merck) as a bacteriostatic agent and 0.001% Tween®-80 (Merck) to prevent the microspheres forming clumps. Release studies were conducted in silanized Erlenmeyer flasks at 37°C and each assay was replicated three times. The samples were collected periodically, 1 ml of the medium was assayed for BI release and replaced by 1 ml of a fresh buffer to maintain sink condition. BI

Table 2
Experimental results: production yield, encapsulation efficiency (E.E.), average particle size (Dvn) and release properties of BI DL-PLA microspheres

Batch	Yield (%)	BI[a] (wt.%)	E.E. (%)	Dvn (μm)	Burst[b] (%)
1 (−1 −1 −1)	73	4.69	55.18	27.11	39.83
2 (1 −1 −1)	50	6.75	79.29	13.01	26.05
3 (−1 1 −1)	59	4.34	17.71	21.22	98.00
4 (1 1 −1)	48.4	15.28	62.39	28.18	60.82
5 (−1 −1 1)	81.2	5.34	63.00	12.90	60.20
6 (1 −1 1)	79.6	4.79	56.35	7.42	68.23
7 (−1 1 1)	61	4.15	16.93	13.23	70.31
8 (1 1 1)	68.20	4.59	18.73	14.86	72.07
9 (−1.68 0 0)	75.60	5.09	30.87	9.74	66.56
10 (1.68 0 0)	84	9.06	54.90	18.92	98.25
11 (0 1.68 0)	88.80	1.76	58.85	10.90	23.33
12 (0 −1.68 0)	55	1.65	5.52	20.01	99.00
13 (0 0 −1.68)	68	2.53	15.36	9.88	33.33
14 (0 0 1.68)	80.8	4.44	26.94	9.16	95.00
15–20 (0 0 0) (S.D.)	85.7 (0.93)	8.55 (0.20)	51.83 (1.20)	22.24 (2.07)	98.00 (1.55)

[a]Experimental core loading.
[b]BI released during the first 6 h of release experiments.

concentration in the release medium was determined by the analytical method described above.

2.7. Insulin labelling

BI was labelled to study its in vivo release, by a Chloramine-T method (Diaz et al., 1996) using Sephadex G-50 fine column and eluting with 0.01 M isotonic PBS (pH 7.5). Fractions of 500 μl were collected and counted in a γ-counter (Cobra Model, Packard).

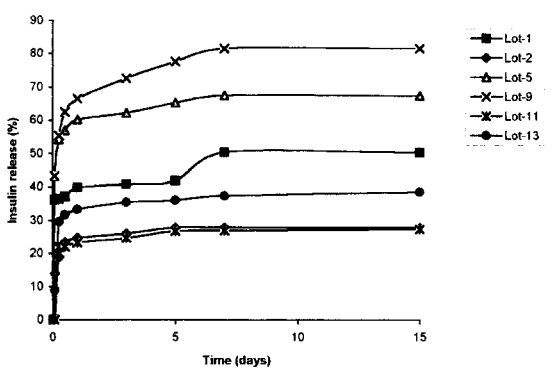

Fig. 2. Cumulative in vitro release of insulin from DL-PLA microspheres in PBS (pH 7.4) at 37°C.

2.8. In vivo release assay

The in vivo study was done injecting subcutaneously a suspension of ^{125}I-BI microspheres in sterile saline 0.5% methyl cellulose (low viscosity) onto the back of six male Wistar rats weighing 250–300 g. The follow up of the amount remaining in the injection site was carried out measuring the radioactivity emitted through an iodine detector (Captus®; Nuclear Ibérica), such as was described in a previous paper (Diaz et al., 1996). Briefly, the release of ^{125}I-BI was followed by periodically accumulating a 1-min spectrum at 27 keV of the injection site; the measurement made at time 0 was considered the injection dose. At different sampling times the radioactivity was measured five times and the median was taken as the right measure.

3. Results and discussion

As already stated, this study was aimed at the evaluation of the influence of the variables (i) polymer molecular weight, (ii) insulin loading and (iii) PVA concentration used in the elaboration process, on microspheres characteristics (produc-

Table 3
Analysis of variance of regression for linear quadratic model used for response surface analysis for each parameter studied

Source of variation	d.f.	Mean squares			
		Burst	E.E.	Dvn	Yield
Total	19				
Linear	3	1830.59[c]	1571[c]	69.70	401.88[c]
Quadratic and cross terms	6	1109.18[c]	163.21	37.91	197.88
Residual	10	269.18	206.59	35.15	2.021
Lack of fit	5	537.92[c]	411.74[c]	66.48[c]	63.16[c]
Pure error	5	0.43	1.45	3.82	0.87
R^{2} [a]		0.82	0.734	0.554	0.84
C.V.[b] (%)		0.67	2.32	8.75	0.67

[a]Calculated correlation coefficients.
[b]Coefficients of variation in relation to the mean of the observations to the central point.
[c]Null hypothesis rejected at $a = 0.05$ level.

tion yield, encapsulation efficiency and particle size) and on the release profile, using a central composite rotable design with three variables.

DL-PLA microspheres prepared were spherical and had pores except for six lots which were non-porous and had smooth surfaces (Fig. 1). Table 2 shows the characteristics of the manufactured microspheres. From in vitro release assays, we observed that the different formulations could be divided into two groups. Ten of them (lots 3, 10, 12, 14, 15–20) released nearly all the incorporated insulin in 24 h. The second group showed a release profile characterized by an initial phase, in which a fast insulin release was produced corresponding to the burst effect, followed by slow, and in some cases null, polypeptide release.

Formulations 4, 6, 7 and 8 were characteristically porous and they presented an elevated burst effect over 40% and reached a maximum of 70% at 48 h; they later went into a latent period for over 15 days, not completing the release. The other six formulations were characteristically non-porous. They were elaborated with all polymers included in the design and had extremely low loading of less than 7%. The formulations 1, 5, 9 and 13 released the polypeptide in a more continuous way, at least during week 1, while lots 2 and 11 reached 28% 5 days after the initiation of the experiment, with a 15% burst effect, but later none of these six formulations completed the release (Fig. 2). This incomplete release seems to be due to the capacity of these polymers to interact with peptides and protein (Bodmer et al., 1992; Sah and Chien, 1993). An infrared Fourier transform (IR-FT) spectrum for diffuse refractance was carried out but no type of chemical interaction was observed since no differences were seen between the spectrums of isolated products and forming microspheres. At the same time, a polypeptide adsorption assay to DL-PLA empty microspheres with molecular weights of 40 000 and 140 000 was carried out and it was observed that the adsorption of insulin is higher with the low polymer molecular weight (40 000); this was expected from results obtained with albumin in a previous work (Soriano et al., 1995). However, the extent of adsorption of BI to the microspheres is lower than protein. Table 3 summarizes the analytical results of analysis of variance (ANOVA) of regression for encapsulation efficiency, particle size, production yield and burst effect (BI released during the first 6 h of in vitro release experiment). The particle size does not depend on any of the variables included in the study; despite the PVA concentration being included in the design as in the literature, contrary references were found. Some authors (Heya et al., 1991; Benoît et al., 1984) pointed out that, on increasing the PVA concentration, the size decreased; others reported contrary findings (Benita

et al., 1984), while Julienne et al. (1992) observed that the PVA concentration did not affect microsphere particle size.

In spite of the fact that the lack of fit was significant (Table 3), the coefficients of variation estimated from the replicated samples of the central point were low (< 2.5%) and the values calculated for the correlation coefficient R^2 were high enough, except for the mean diameter. These data justify the use of the quadratic model for the analysis of the response surfaces.

For the response surfaces studies, we used the Wolfram Mathematica™ program (Wolfram, 1988). The response surface for the trapping efficiency (Fig. 3a) shows a saddle point (0.443, −2.043, 0.031) around the experimental point 90 000–120 000 (M_w), less than 3% BI (which is out of the range studied) and 0.13% PVA. The quadratic equation for the burst effect, the parameter chosen to evaluate the polypeptide release, gives a response surface showing a maximum on the point (0.237, 0.433, 0.343) (Fig. 3b),

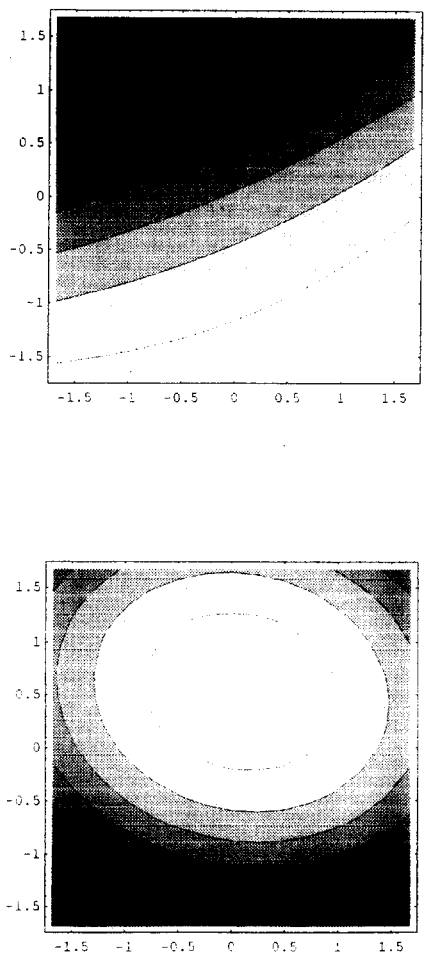

Fig. 3. Response surfaces as function of coded variables molecular weight of polymer (x-axis) and insulin content (y-axis): (a) encapsulation efficiency; (b) burst effect.

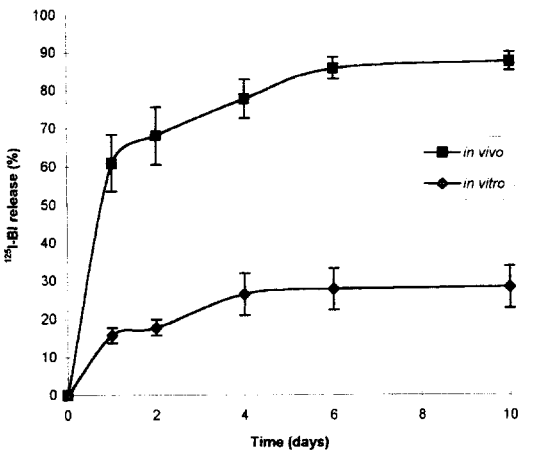

Fig. 4. In vitro–in vivo comparison of ^{125}I-BI released from DL-PLA microspheres.

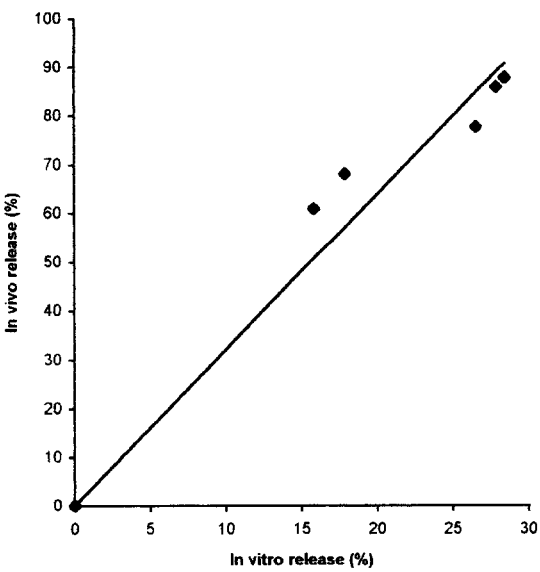

Fig. 5. In vitro–in vivo correlation of ^{125}I-BI released from DL-PLA microspheres.

while the minimum is located around low molecular weight, low and high insulin loading and 0.01–0.13% PVA concentration.

From the results obtained with the experimental design, we can conclude that, as shown by the model equation, a larger burst effect and less encapsulation efficiency is observed when insulin content increases. This could be due to the fact that during the evaporation process, a large part of insulin is lost and channels or pores are originated in the microsphere structure.

Taking into account the results of the burst effect and encapsulation efficiency from the model (in which the optimum conditions are found in the area of low insulin loading and polymer molecular weight, with a PVA concentration of 0.01 and 0.13%), a new lot with DL-PLA of M_w 60 000, 3% BI mixed with ^{125}I-BI and 0.01% of PVA was elaborated and tested in vitro and in vivo. In this lot, the encapsulation efficiency was 75%. The in vitro release was carried out in the same conditions as were explained above. The amount of ^{125}I-BI released was measured after the samples were centrifuged and supernatants counted in the γ-counter. Fig. 4 compares the in vitro and in vivo release profiles of ^{125}I-BI; as the model predicts, the burst effect was lower (5%) in these microspheres prepared at lower insulin content and lower PVA concentration, but we also observed a decrease in the release rate and more incomplete release (28%), at least in the 10 days that the assay lasted. The in vivo assay is characterized by a high release (60%) in the first 24 h, while about 90% of the dose is released in the following 10 days. In Fig. 5, a good in vitro–in vivo correlation is shown; as can be seen, the in vivo release is 3-fold higher than the in vitro one.

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