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Release control of albumin from polylactic acid microspheres

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Abstract

This paper reports how poly(DL-lactide) microspheres of different molecular weight and BSA content were manufactured in order to evaluate the influence of these two variables on the protein release profile, using a composite central rotational design. The microspheres were prepared by a double emulsion method using different w/o phase ratios. The BSA encapsulated was about 80% of the theoretical amount incorporated. The resulting microspheres proved porous except for three batches. The release of BSA from microspheres presented two phases, first a high burst effect ranging from 37.3 to 75% of the protein incorporated, and a second slower one. Analysis of the release results clearly shows a positive linear dependence of the slope of the slow release phase on the molecular weight of polymer, due most likely to the formation of hydrogen bridges between the terminal -OH groups of the polymer chains and the solitary pairs of donor atoms of the protein. Non-porous microspheres with a lesser burst effect were obtained by decreasing the w/o ratio of the first emulsion during the preparation process.

Keywords: Microsphere; Poly(DL-lactic) acid; (w/o)/w emulsion; Bovine serum albumin; Sustained release; Initial burst effect

1. Introduction

The use of biodegradable polymers to manufacture microspheres which release peptides over extended periods of time has been investigated in recent years and satisfactory results have been obtained, principally for LHRH agonists (Ogawa et al., 1988a–c; Okada et al., 1989) and TRH (Heya et al., 1991). The potential of proteins as therapeutic agents is increasingly being recognised, however, these drugs have high molecular weights and marked solubility in addition to their special structure so that the experience gained through peptides does not necessarily apply to them.

Albumin has been selected as the experimental protein for the manufacture of poly(lactide-co-glycolide) microspheres and remains stable throughout both the preparation facture and release processes (Hora et al., 1990; Cohen et al., 1991). The influence on the release kinetics of sundry variables, such as the effect of the homogenization techniques used in manufacture, the composition and biodegradation of the copolymers, the amount of incorporated drug and the characteristics of the release medium, have been evaluated (Sah and Chien, 1993).

The release mechanisms of this type of macromolecule have not been elucidated and it is suggested that release from systems manufactured

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with biodegradable poly(lactide-co-glycolide) type polymers takes place in two or three phases: the first one, a burst effect, corresponding to the release of the non-encapsulated product close to the surface, the second, the formation of pores or channels in the matrix due to polymer degradation and the third, bioerosion (Furr and Hutchinson, 1992).

This paper reports how poly(DL-lactide) microspheres with different molecular weights and albumin content were manufactured in order to evaluate the influence of these two variables on the protein release profile, using a central composite rotable design.

2. Material and methods

DL-PLA of different molecular weights was obtained according to the ring-opening reaction described by Kulkarni et al. (1966). The experimental synthesis conditions, time, temperature and catalyst concentration of tetraphenyl tin (Merck) 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 of various molecular weights were used (Tokyo Soda Ltd). Polyvinyl alcohol (PVA) of average molecular weight 30 000–70 000 was purchased from Sigma Chemical Co. Bovine serum albumin (BSA) came from Merck. All the other chemicals were reagent grade.

2.1. Experimental design

The following quadratic model was used,

$$y = b_0 + \sum_{2} b_i x_i + \sum_{i \le j} b_{ij} x_i x_j + \epsilon$$

where $i = 1,2$ for the variables weight average molecular weight of polymer (M_w) and drug content, respectively, Y denotes the response and ϵ is the error, which is assumed to be $N(0,\sigma^2)$.

A central composite rotatable design was used which contained nine different combinations of the variables and the central point was replicated five times (Cochran and Co, 1980). Table 1 shows the coded levels and values of design variables.

Table 1
Coded levels corresponding to the central composite rotational design for polymer molecular weight (X_1) and initial percentage of albumin (X_2)

Level	X_1	X_2
−1.414	40 000	16.7
−1	55 000	21.5
0	90 000	33.3
1	125 000	45
1.414	140 000	50

2.2. Microsphere preparation

PLA microspheres were prepared by a double emulsion method which is a modification of that described by Ogawa et al. (1988a–c). BSA was dissolved in water (1 ml) and poured into a solution of DL-PLA in $MeCl_2$ (Merck). The mixture was sonicated for 1 min to obtain the first emulsion (w/o). The DL-PLA concentrations in the organic phase were from 66.6 to 111 mg/ml, the phase ratios ($H_2O/MeCl_2$) being from 1:4 to 1:7. The first emulsion was poured into 400 ml of aqueous 0.1% PVA and the solution was stirred with a homogenizer (Ultraturrax® T-25) at 8000 rpm at 5°C and stirred for 10 min to make a (w/o)/w emulsion which was then stirred for 3 h at room temperature at 250 rpm to remove the solvent from the microspheres which had formed. The microspheres were collected by filtration through an aqueous Millipore® filter (0.45 μm) and dried in vacuum for 24 h.

The albumin content of the microspheres was determined by dissolving them in dichloromethane and extracting the albumin in water. The concentration of BSA was measured by the spectrophotometric method of Lowry et al. 1951).

2.3. In vitro release study

Microspheres (40–50 mg) were suspended in isotonic PBS (50–100 ml), pH 7.4, containing 0.02% sodium azide as a bacteriostatic agent and

0.001% Tween® −80 to prevent the microspheres forming clumps. Release studies were conducted in silanized Erlenmeyer flasks at 37°C and each assay was replicated three times. At each time interval 1 ml of the medium was withdrawn and albumin release was determined by the analytical method described above, the suspension being refilled with 1 ml of fresh medium.

2.4. Particle size

Microspheres suspended in 0.9% NaCl solution were measured using a Coulter counter (Coulter® Multisizer II) after treatment in a bath sonicator for 3 min to bring about disaggregation.

2.5. Morphology

The shapes and surface characteristics of the dried microspheres were examined by scanning electron microscopy (SEM). To study their surface structure, the microspheres were coated with gold/palladium under an argon atmosphere.

3. Results

Morphological characteristics: the prepared microspheres proved porous except for batches A, E and G which were virtually non-porous and smooth-surfaced. Fig. 1a and b shows microspheres from batches A and F as examples of non-porous and porous microspheres, respectively.

Table 2 sets out the characteristics of the manufactured microspheres. Batches I, I1, I2, I3 and I4 correspond to the central point and its replicates. As can be seen in Table 2, the manufacturing method is reproducible, with values for production of between 89.2 and 90.6%, and from

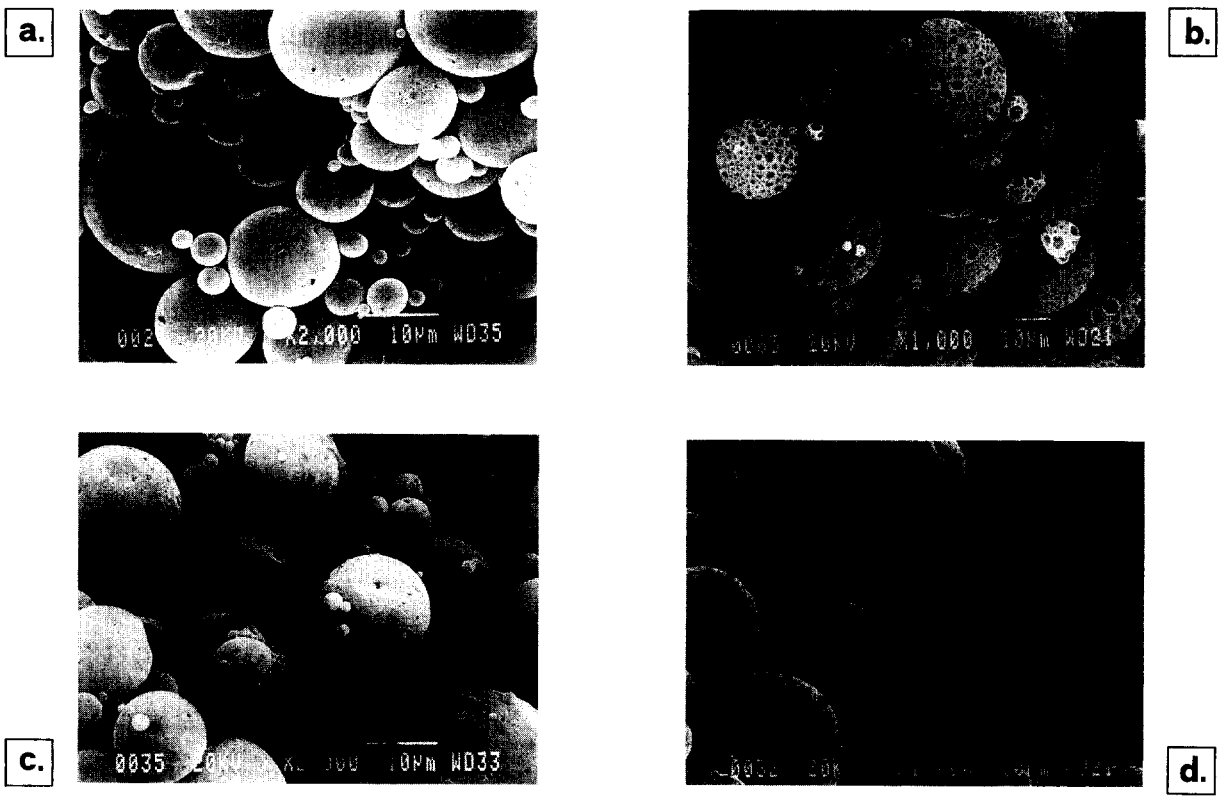

Fig. 1. Scanning electron micrograph of BSA microspheres prepared by double-emulsion technique: lot A (a,c), lot F (b,d). Immediately after preparation (a,b) and after release assay (c,d). Bar represents 10 μm.

Table 2
Experimental results: production yield, BSA entrapping efficiency (E.E.) and mean volume diameter (dvn)

Batch	Production (%)	BSA [a] (%)	E.E. (%)	dvn (μm)
A (−1,−1)	80.1	11.31	52.7	23.9
B (1,−1)	84.3	15.28	71.0	30.3
C (−1,1)	79.9	20.94	46.5	32.7
D (1,1)	78.9	33.72	74.9	22.7
E (−1.4,0)	82.4	6.90	20.8	20.9
F (1.4,0)	87.3	30.41	91.3	31.8
G (0,−1.4)	82.5	7.17	43.0	26.8
H (0,1.4)	82.1	21.57	43.1	23.3
I1–I5 (0,0)	89.9	26.81	79.9	32.2
(SD)	(0.59)	(0.77)	(0.76)	(2.24)

[a] Experimental BSA loading.

Table 3
Results of BSA release from the microspheres

Batch	Burst (%)	$K(\times 10^2)$ (h^{-1})	X_{max} (%)	Time (h)
A	49.9	3.87	77	720
B	74.1	55.20	92	72
C	61.9	2.57	81	720
D	67.4	44.10	77	72
E	42.6	5.85	64	360
F	46.6	27.40	80	192
G	57.9	10.30	88	360
H	37.3	3.19	54	720
I1–I5	60.1	3.30	89.40	720
(SD)	(1.42)	(9.59×10^{-4})	(2.65)	

Burst effect and release rate (K) were obtained by linear regression. Duration of release assay in vitro and BSA release percentage are given.

79.0 to 81.0% for trapping. The mean diameters ranged from 30.9 to 35.3 μm and all batches were spherical in form and had porous surfaces.

In the in vitro release assay, the albumin in all the batches of microspheres was delivered in two stages: an initial rapid one which corresponds to the so-called burst effect and a second, slower phase. Table 3 shows the burst effect and release rate for each of the lots studied, estimated by linear regression from the slower phase cumulative profile, the intercept corresponding to the burst effect (lot B was the maximum with 75% and lot H the minimum with 37% (Fig. 2)) and the slope to the release rate.

Table 4 summarizes the analytical results of analysis of variance (ANOVA) of regression for trapping, microsphere diameter, burst effect and slope of the slow release phase (release rate). As can be seen, both the percentage entrapped and the release rate depend linearly on the molecular weight and the theoretical amount of BSA incorporated; on the other hand, the burst effect does

Table 4
Analysis of variance of regression for linear quadratic model used for response surface analysis for each parameter

Source of variation	df	Mean squares				
		Burst	Release rate	E.E.	dvn	Yield
Total	12					
linear	2	113.88	0.098 [c]	1345 [c]	0.098	8.96
Quadratic and cross terms	3	137.406	0.029	679.11 [c]	67.87 [c]	65.72 [c]
Residual	7	118.38	0.013	89.92	10.66	3.19
lack of fit	3	270.45 [c]	0.030 [c]	209.03 [c]	18.15	7.001 [c]
Pure error	4	4.32	49.10^{-7}	0.58	5.039	0.34
R^2 [a]		0.44	0.756	0.883	0.749	0.906
C.V. [b] (%)		3.206	2.906	0.96	6.76	0.65

[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 $\alpha = 0.05$ level.

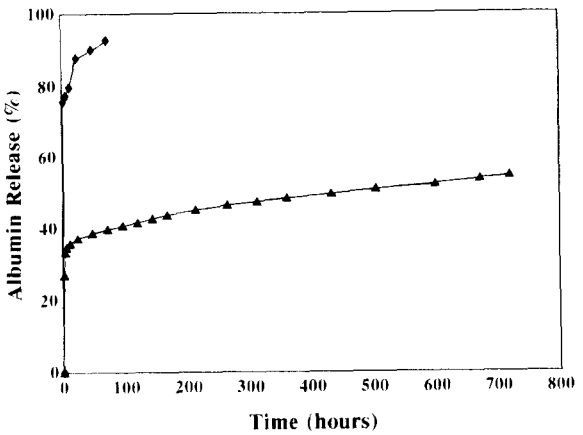

Fig. 2. Cumulative in vitro release of BSA from DL-PLA microspheres in PBS (37°C): lot B (♦) and lot H (▲).

not depend on any of the variables of the experimental design. For the mean diameter and the percentage of trapping, the null hypothesis was rejected for the quadratic and cross terms. Table 5 lists the estimated coefficients of quadratic model used for response surface analysis.

4. Discussion

In spite of the fact that, except for the mean diameter, the lack of fit was significant (Table 4), the coefficients of variation estimated from the replicated samples of the central point, were low, and the calculated correlation coefficients R^2 were high enough, however, for the burst effect. These data justify the use of the quadratic model for the analysis of the response surfaces.

Table 5
Estimated coefficients of quadratic model used for response surface analysis

Coefficient	Burst	Release rate	E.E.	dvn	Yield
b_0	64.85	0.033	79.902	33.16	89.92
b_1	4.42	0.15 [a]	18.34 [a]	1.46	1.27
b_2	−2.98	−0.028	−0.25	−0.47	−0.78
b_{11}	−5.81	0.103 [a]	−9.004 [a]	−3.013 [a]	−3.21 [a]
b_{22}	−4.32	0.054	−15.48 [a]	−3.61 [a]	−4.508 [a]
b_{12}	−4.67	−0.024	2.47	−4.092 [a]	−1.31

[a] Null hypothesis rejected at $\alpha = 0.05$ level.

The production yield presents a maximum on the point (0.22–0.119), corresponding approximately to the experimental point of 90 000 for the molecular weight of polymer and 33% of BSA were very close to the maximum for the mean diameter which corresponds to the area of 90 000–125 000 (M_w) and 33–45% of BSA.

The quadratic equation for the trapping efficiency gives the response surface shown in Fig. 3 and has a maximum on the point (1.03–0.09) around the experimental point 125 000 (M_w) and 33% of BSA.

Analysis of the results clearly shows a positive linear dependence of the slope of the slow release phase, release rate, on the molecular weight of the polymer; the greater the M_w the quicker the release of the drug (Fig. 4), a finding completely at odds with the results published for most conventional drugs (Cha and Pitt, 1988; Bodmeier et al., 1989; Jalil and Nixon, 1990) and peptides (Wada et al., 1990; Asano et al., 1991; Yamakawa et al., 1992). This effect may only be appreciated when studies with polymers are carried out with a wide range of molecular weights,

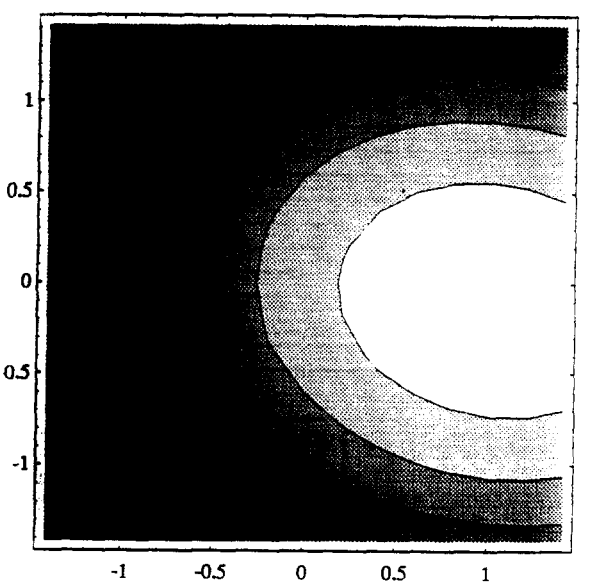

Fig. 3. Response surface of the trapping efficiency as a function of coded variables molecular weight of polymer (x-axis) and drug content (y-axis).

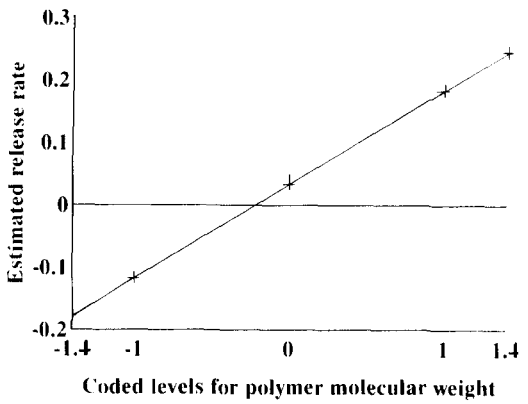

Fig. 4. Linear dependence of the release rate on the molecular weight of the polymer.

such as the design used in this paper where molecular weights ranged from 40 000 to 140 000 or the study published by Bodmer et al. (1992) in which the same phenomenon was recorded for octapeptide (Octrotide) microspheres and cylinders containing albumin manufactured with poly(DL-lactide-co-glycolide), a more hydrophilic material than the DL-PLA homopolymer. The range of molecular weights studied by these researchers was 23 000–74 000. This effect cannot be detected in studies made with polymers of molecular weights close to or under 20 000. Protein release in this second phase, as mentioned above, has been attributed to the formation of channels in the matrix due to polymer degradation, but determination of the molecular weight of the microspheres once the release assay was over showed it to be unchanged and, as can be seen from Fig. 1c and d the microspheres retained their original structure, indicating that the polymer does not need to be degraded for albumin to be considerably released from these microspheres. Hence, the most likely mechanism would be penetration of the medium through the pores initially present in the microspheres or those formed by erosion or albumin dissolution and release. However, if this were the mechanism at work, it would be expected that lots A, E and G with very few superficial pores and low dose uptake (Table 2) would release albumin more slowly than the others, all of which were very porous and contained more drug. As can be seen in Table 3, this is not the case and the lot which displays the least burst effect is lot H (37.3%) while lot C is the one with the smallest slope in the second phase (0.0257 h^{-1}). Nonetheless, it is noteworthy that lot E, consisting of non-porous microspheres of low molecular weight (40 000), did not release its full complement, at least not within the fortnight that the assay lasted.

Another of the factors that may affect albumin release from poly(DL-lactide-co-glycolide) microspheres is the volume of internal aqueous and oily phase (Ogawa et al., 1988a–c; Jeffery et al., 1993). It has been noted that there is less burst effect when the volume of aqueous phase is decreased (Sah and Chien, 1993) and if the concentration of the polymer in the organic phase is raised at the same time (Cohen et al., 1991), both burst effect and release rate are reduced. Lots A, E and G were prepared with high polymer concentration (104.6, 97 and 111 mg/ml, respectively), giving non-porous microspheres.

To obtain microspheres with a denser core which hinders penetration of the medium and delays release, three lots, A′, B′ and G′ were prepared under the same conditions as lots A, B and G but reducing the volume of organic solvent from 6 to 3 ml which supposed double polymer concentration. An important reduction in the rate of the second phase and burst effect was observed except in the case of lot B′ where the burst effect was not greatly affected (Fig. 5). The three batches were all non-porous. As in the previous design, the lower the molecular weight, the smaller the slope and in this instance also the lesser the burst effect (lot A). It should also be noted that lot B′ took the whole fortnight that the assay lasted to complete the release sequence. All this suggests that the ratio of aqueous/oily phase, and especially the polymer concentration in the organic solvent, in the first emulsion owing to its viscosity makes an important contribution to the characteristics of the internal structure of the microspheres, which also influence protein release.

From this study it can be gathered that the in vitro albumin release from microspheres made of poly(DL-lactic acid) depends on the amount of trapped drug, the polymer concentration in the

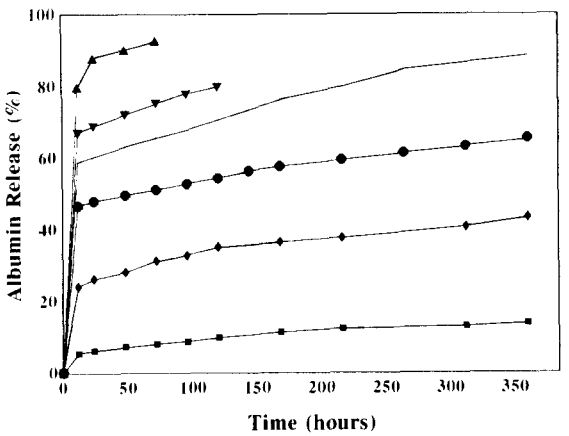

Fig. 5. Cumulative in vitro release of BSA from DL-PLA microspheres in PBS (37°C): effects of phase relationship; lot A (●), lot B (▲), lot G (*), lot A′ (■), lot B′ (▼) and lot G′ (◆).

organic phase and the molecular weight of the polymer. Polymers with lower molecular weight show a smaller amount released due, most likely as has been suggested by Bodmer et al. (1992) and Sah and Chien (1993), to the presence of hydrogen bridges between the terminal OH groups of the polymer chains, which are more numerous than in high molecular weight polymers, and the isolated pairs of donor atoms of the protein.

To verify this possible explanation an FT-IR spectrum by diffuse refractance was recorded, no type of chemical binding seeming to be produced,

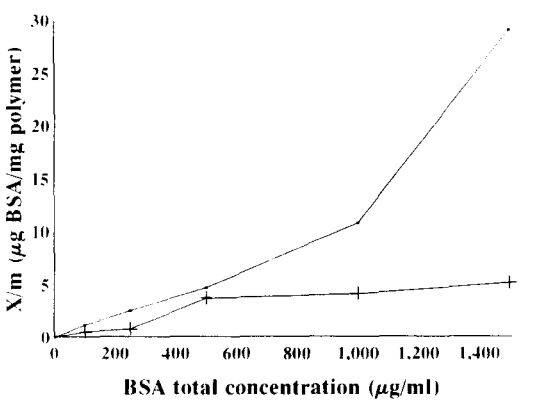

Fig. 6. Amount of BSA adsorbed by polymer mass unit vs total BSA concentration in the medium.

since no differences between isolated products and forming microspheres could be appreciated.

An assay of protein adsorption to empty polymer microspheres was performed. Albumin free microspheres (10 mg) were suspended in different solutions of BSA in the same medium used for the in vitro release studies. The BSA concentration varied from 100 to 1500 μg/ml and the assay was carried out at 37°C in silanized tube under slow stirring. DL-PLA (M_w 40000) was able to adsorb a greater amount of albumin (6-fold more) than micrsopheres prepared with high molecular weight DL-PLA (M_w 140000) (Fig. 6), which could justify the different behaviour in protein transfer.

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References

Asano, M., Fukuzaki, H., Yoshida, M., Kumakura, M., Mashimo, T., Yuasa, H., Imai, K., Yamakana, H., Kawahara, V. and Suzuki, K., In vivo controlled release of a luteinizing hormone-releasing hormone agonist from poly(DL-lactic acid) formulations of varying degradation pattern. *Int. J. Pharm.*, 67 (1991) 67–77.

Bodmeier, R., Oh, K.H. and Chen, H., The effect of the addition of low molecular weight poly(DL-lactide) on drug release from biodegradable poly(DL-lactide) drug delivery systems. *Int. J. Pharm.*, 51 (1989) 1–8.

Bodmer, D., Kissel, T. and Traechslin, E., Factors influencing the release of peptides and proteins from biodegradable parenteral depot systems. *J. Controlled Release*, 21 (1992) 129–138.

Cha, Y. and Pitt, C.G., A one-week subdermal delivery system for L-methadone based on biodegradable microcapsules. *J. Controlled Release*, 7 (1988) 69–78.

Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H. and Langer, R., Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. *Pharm. Res.*, 8 (1991) 713–720.

Cochran, W.G. and Cos, G.M., *Diseños Experimenntales*, Trillas, México, 1980, p. 372.

Furr, B.J.A. and Hutchinson F.G., A biodegradable delivery system for peptides: preclinical experience with the go-

nadotropin-releasing hormone agonist Zoladex®. *J. Controlled Release*, 21 (1992) 117–128.

Heya, T., Okada, H., Ogawa, Y. and Toguchi, H., Factors influencing the profiles of TRH release from copoly(d,l-lactic/glycolic acid) microspheres. *Int. J. Pharm.*, 72 (1991) 199–205.

Hora, M.S., Rana, R.K., Nunberg, J.H., Tice, T.R., Gilley, R.M. and Hudson, M.E., Release of Human Serum Albumin from poly(lactide-co-glycolide) microspheres. *Pharm. Res.*, 7 (1990) 1190–1194.

Jalil, R. and Nixon, J.R., Microencapsulation using poly(DL-lactic acid): II. Effect of polymer molecular weight on the microcapsule properties. *J. Microencapsul.*, 7 (1990) 245–254.

Jeffery, H., Davis, S.S. and O'Hagan D.T., The preparation and characterization of poly(lactide-co-glycolide) microparticles: II. The entrapment of a model protein using a (water-in-oil)-in-water emulsion solvent evaporation technique. *Pharm. Res.*, 10 (1993) 362–368.

Kulkarni, R.K., Pani, K.C., Newman, C. and Leonard, F., Polylactic acid for surgical implants. *Arch. Surg.*, 93 (1966) 839–843.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurements with the Folin-Phenol reagent. *J. Biol. Chem.*, 193 (1951) 265–275.

Munguía, O., Delgado, A., Fariña, J., Evora, C. and Llabres M., Optimization of dl-PLA molecular weight via the response surface method. *Int. J. Pharm.*, 86 (1992) 107–111.

Ogawa, Y., Okada, H., Yamamoto, M. and Shimamoto, T., In vivo release profiles of leuprolide acetate from microcapsules prepared with polylactic acids or copoly(lactic /glicolic) acids and in vivo degradation of these polymers. *Chem. Pharm. Bull.*, 36 (1988a) 2576–2581.

Ogawa, Y., Yamamoto, M., Okada, H., Yashiki, T. and Shimamoto, T., A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly(lactic/glycolic) acid. *Chem. Pharm. Bull.*, 36 (1988b) 1095–1103.

Ogawa, Y., Yamamoto, M., Takada, S., Okada, H. and Shimamoto, T., Controlled-release of leuprolide acetate from polylactic acid or copoly(lactic/glycolic) acid microcapsules: Influence of molecular weight and copolymer ratio of polymer. *Chem. Pharm. Bull.*, 36 (1988c) 1502–1507.

Okada, H., Heya, T., Igari, Y., Ogawa, Y., Toguchi, H. and Shimamoto T., One-month release injectable microspheres of leuprolide acetate inhibit steroidogenesis and genital organ growth in rats. *Int. J. Pharm.*, 54 (1989) 231–239.

Sah, H. and Chien, Y.W., Evaluation of a microreservoir-type biodegradable microcapsule for controlled release of proteins. *Drug Dev. Ind. Pharm.*, 19 (1993) 1243–1263.

Wada, R., Hyon, S.H. and Ikada Y., Lactic acid oligomer microspheres containing hydrophilic drugs. *J. Pharm. Sci.*, 79 (1990) 919–924.

Yamakawa, I., Tsushima, Y., Machida, R. and Watanabe, S., In vitro and in vivo release of poly(DL-lactic acid) microspheres containing Neurotensin analogue prepared by novel oil-in-water solvent evaporation method. *J. Pharm. Sci.*, 81 (1992) 808–811.