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Somatostatin containing biodegradable microspheres prepared by a modified solvent evaporation method based on W/O/W-multiple emulsions

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Abstract

Biodegradable polyester microspheres containing somatostatin acetate, a peptide drug, were prepared by a modified solvent evaporation method based on the formation of multiple W/O/W-emulsions. The resulting microspheres were characterized with respect to drug loading, encapsulation efficiency and morphological characteristics. Various methods for extracting the peptide from the microspheres were compared. Of all parameters investigated, factors affecting the properties of the primary W/O-emulsion, such as the phase volume ratio and total volume, were of major importance. A small volume of internal aqueous phase and an intermediate volume of organic solvent were favorable to achieve high drug encapsulation efficiencies. Replacing methylene chloride as an organic solvent with ethyl acetate reduced the encapsulation efficiency and resulted in more porous microspheres. Except for microspheres prepared with very low molecular weight polymers, the encapsulation efficiency was not affected by the polymer type (poly(L-lactide), poly(D,L-lactide), poly (D,L-lactide/glycolide)) and molecular weight. The preparation conditions substantially affected the morphology and porosity of the microspheres.

Keywords: Biodegradable polymer; Microencapsulation; Microspheres; Peptide delivery system; Poly(lactide); Solvent evaporation method; Somatostatin

1. Introduction

Peptide drugs have attracted considerable medical and pharmaceutical interest. Despite intense investigations of alternative routes of administration (e.g. oral, nasal, transdermal, colonic), the parenteral route is predominant because of short in-vivo half-lives, poor bioavailability, and poor stability (Lee, 1991). Controlled release parenteral dosage forms are needed to improve the efficacy of these drugs by reducing the frequency of injections and by decreasing plasma level fluctuations. Somatostatin (growth hormone release inhibiting factor, SRIF), the peptide used in this study, is a

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typical representative of a biologically active peptide drug with high therapeutic potential, but short duration of action (the in-vivo half-life after intravenous injection is 1.1–3.0 min (Lee, 1991)).

Encapsulation with biodegradable polymers represents one way of overcoming various problems associated with the delivery of peptides. The microparticles can release the drug in a controlled manner according to specific therapeutic requirements. Poly(D,L-lactide) and its glycolic acid copolymers are the most widely used materials. They are well-characterized, have good biocompatibility, and are approved for parenteral use by the FDA.

Injectable microspheres can be prepared by several techniques, with solvent evaporation and organic phase separation methods being used most frequently. In the solvent evaporation method the drug is dissolved, dispersed or emulsified into an organic polymer solution, which is then emulsified into an external aqueous phase to form droplets (Bodmeier et al., 1991). The microspheres are formed after solvent removal and polymer precipitation. In the phase separation method, polymer precipitation around a dispersed drug-containing phase is induced by the addition of a non-solvent or a temperature change, leading to the formation of microcapsules (Ruiz et al., 1989).

Extensive research in this field has resulted in several publications describing the preparation of peptide-containing biodegradable microspheres and their in vitro and in vivo characterization. Especially solvent evaporation and phase separation techniques have been used to encapsulate LH-RH antagonists and other peptide or protein drugs in polymeric microspheres (Sanders et al., 1986; Mason-Garcia et al., 1988; Ogawa et al., 1988; Ruiz et al., 1989; Csernus et al., 1990; Cohen et al., 1991; Heya et al., 1991). However, only little information is available on the principal factors affecting the efficiency of these methods. It was the objective of this study to systematically investigate preparation parameters of a modified solvent evaporation method based on the formation of a W/O/W-emulsion with respect to drug loading, encapsulation efficiency and structural properties of the resulting microspheres.

2. Materials and methods

2.1. Materials

The following materials were used as received: somatostatin acetate (Dr. Willmar Schwabe Arzneimittel, Karlsruhe, Germany), poly(L-lactide) (L-PLA, M.W. 94000), poly(D,L-lactide) (D,L-PLA, M.W. 110000), poly (D,L-lactide/glycolide) (PLGA 85/15, M.W. 87000 and 6400; PLGA 50/50, M.W. 53000) (Medisorb Technologies Ltd., Cincinnati, OH), poly(D,L-lactide) (D,L-PLA, Resomer R104 and R202; M.W. 2000 and 6000, Boehringer Ingelheim, Ingelheim, Germany), poly(vinyl alcohol) (PVA, 88 mol% hydrolyzed, M.W. 125000, Polysciences, Inc., Worthington, PA), acetone, methylene chloride, phosphoric acid (EM Science, Gibbstown, NJ), ethanol (AAPER, Shelbyville, KY), ethyl acetate (Mallinckrodt, Paris, KY), acetonitrile (Fisher Scientific, Fair Lawn, NJ).

2.2. Methods

The solubility of somatostatin acetate in various organic solvents was measured by adding excess drug to 1.0 ml of solvent; the resulting drug suspensions were agitated for 24 h at room temperature in glass vials sealed with Teflon lined screw caps; 0.5 ml of the supernatant was removed after centrifugation and evaporated in a nitrogen stream. The residue was dissolved in 0.5 ml water and analyzed by HPLC (described below) to determine the drug solubility. The approximate solubilities in 10 mM pH 5.9 acetate buffer, methanol, and ethanol were obtained during the preparation and extraction of microspheres under various conditions.

The microspheres were prepared by a modified solvent evaporation method based on the formation of a W/O/W-emulsion (Ogawa et al., 1988; Alex and Bodmeier, 1990). An aqueous solution of somatostatin acetate was emulsified into a solution of the polymer in a water-immiscible solvent or solvent mixture by vortex mixing followed by sonication for 30 s under ice-cooling (Heat Systems, Ultrasonics, Inc., Plainview, NY). Sonication had no negative effect on the peptide

stability. The resulting primary W/O-emulsion was then injected with a disposable syringe into the external aqueous phase under continuous stirring with a magnetic stirrer (Corning PC-351 hot plate stirrer, Corning, Inc., NY) at room temperature and ambient pressure. After 30 min (90 min for low molecular weight polymers D,L-PLA 2000, 6000 and PLGA 85/15 6400), the microspheres were collected by filtration, rinsed with 30 ml water, vacuum-dried, sieved (US Standard Sieve Series, Dual MFG, CO., Chicago, IL), and stored in a desiccator. The 75–180 μm particle size fraction was used for further investigations. The standard conditions for the microsphere preparation and the investigated ranges of the formulation and process variables are summarized in Table 1.

In order to obtain the actual drug loading, three different methods for the extraction of somatostatin from the microspheres were evaluated. In the first method, drug-loaded microspheres (5 mg) were dissolved in 1.0 ml methylene chloride

Table 1
Standard preparation parameters and their investigated range

Preparation parameter	Standard	Investigated range
Polymer	PLGA 85/15, M.W. 87 000	Table 3
Amount of polymer (mg)	150	75–400
Theoretical drug loading (%w/w)	1.9	1.0–10.0
Internal aqueous phase	10 mM pH 5.9 acetate buffer	–
Volume of internal aqueous phase (ml)	0.10	0.05–0.50
Organic solvent composition	CH_2Cl_2	Ethyl acetate, CH_2Cl_2/acetone, 10:0–7:3 v/v
Volume of organic phase (ml)	3.0	1.0–6.0
Volume of external aqueous phase (ml)	500	100–800
PVA concentration (%w/v)	0.25	0–1.0
Stirring time (h)	0.5	0.25–24

followed by the addition of 1.0 ml of 1 M acetic acid and gentle agitation for 12 h at room temperature (CH_2Cl_2/acetic acid method) (Ruiz et al., 1989). The low pH of the aqueous phase had no detrimental effect on the stability of the drug (Herrmann and Bodmeier, 1993). This method was used as the standard extraction method. In the second method, microspheres (5 mg) were extracted with 1.5 ml methanol for 12 h at room temperature (methanol method) (Bodmeier et al., 1991). In the third method, the microspheres were dissolved in an acetonitrile/water mixture (0.5 ml, 9:1 v/v) followed by precipitation of the polymer with 1.0 ml of 10 mM pH 6.0 acetate buffer (acetonitrile/acetate buffer method) (Sanders et al., 1986). The somatostatin content of the microspheres was determined by analyzing the respective somatostatin solution with a stability sensitive HPLC method: LC-600-HPLC-pump, SIL-9A autoinjector, SPD-6A UV-detector, CR-601 integrator (Shimadzu, Kyoto, Japan); ET 250/8/4, Nucleosil 300-5 C18 column (Macherey and Nagel, Düren, Germany); Vydac I-218TP guard column (Vydac, Hesperia, CA); mobile phase: 75.5% v/v water, 24.0% v/v acetonitrile, 0.5% v/v phosphoric acid; flow rate: 0.9 ml/min; UV-detection at $\lambda = 210$ nm. Somatostatin solutions of known concentrations (0.01–0.15 mg/ml) in the respective solvent systems were used to generate calibration curves. This method was validated with respect to linearity ($r > 0.999$), sensitivity (2 μg/ml), precision (3% RSD) and accuracy (approx. 10% RSD) (Debesis et al., 1982).

To study the surface properties and the internal structure of the microspheres, the particles were coated for 70 s with gold palladium (Pelco Model 3 Sputter Coater) and observed with a scanning electron microscope (SEM) (Jeol JSM 35C). Cross-sections were obtained by dispersing the microspheres in a glue (Testor Corporation, Rockford, IL) followed by cutting of the dried matrix with a razor blade prior to coating.

3. Results and discussion

The solvent evaporation method is a popular microencapsulation technique for the preparation

Table 2
Solubility of somatostatin acetate in various solvents

Solvent	Solubility (mg/ml)
10 mM pH 5.9 acetate buffer	> 70
Methanol	> 15
Ethanol	6
Ethyl acetate	$< 2 \cdot 10^{-3}$
Acetone	$< 2 \cdot 10^{-3}$
Acetonitrile	$3 \cdot 10^{-2}$
Methylene chloride	$< 2 \cdot 10^{-3}$

of drug-containing matrix particles from water-insoluble polymers. Compared with other microencapsulation methods, the solvent evaporation process shows greater flexibility with respect to the solubility of the drug to be encapsulated. Various modified techniques based on aqueous or non-aqueous external phases allow the encapsulation of lipophilic as well as hydrophilic drugs.

In this study, a technique based on the formation of a multiple W/O/W emulsion was selected for the encapsulation of somatostatin acetate, a water-soluble peptide. A primary W/O emulsion consisting of an aqueous drug solution emulsified into a solution of the polymer in a water-immiscible solvent was emulsified into an external aqueous phase to form a W/O/W emulsion. The microspheres formed after solvent diffusion/evaporation and precipitation (hardening) of the polymer in the emulsified droplets. Key parameters for the successful encapsulation of the drug are a small droplet size of the internal aqueous phase and the insolubility of the drug in the organic polymer solution; the organic polymer solution acts as a barrier between the internal drug-containing aqueous phase and the continuous aqueous phase. Due to the high potency of the drug, the theoretical drug loading used in this study was low (1.9% w/w).

In order to find suitable solvents, the solubilities of somatostatin acetate in various solvents were determined (Table 2). Somatostatin acetate was very soluble in hydrophilic solvent systems such as acetate buffer or methanol. The high solubility and the high cost of the drug prevented the preparation of saturated solutions in these solvents. The peptide was insoluble in methylene chloride and ethyl acetate (solubility less than 2 μg/ml), two organic solvents suitable for this microencapsulation process; they were good solvents for the biodegradable polymers and water-immiscible, thus allowing the emulsification of the polymer solution.

Important parameters in the evaluation of a microencapsulation technique are the actual drug loading, the encapsulation efficiency, the yield, the batch-to-batch reproducibility, morphological characteristics of the microspheres, and solvent residuals of the final product. The major parameter used in this study for the characterization of the microsphere formulation was the encapsulation efficiency. This parameter was calculated from the experimentally determined actual drug loading of the microspheres and the theoretical drug loading. Considering the high price of peptide drugs, microencapsulation processes with high encapsulation efficiencies and yield are desirable.

In order to obtain accurate and precise data on the actual somatostatin acetate contents and encapsulation efficiencies of the microspheres, suitable extraction and analytical methods had to be developed. The extraction of polymer films containing known amounts of drug, with a methylene chloride/acetic acid mixture yielded acceptable drug recovery (101.5%) and reproducibility ($\pm$ 4.6%, $n = 4$), independent of the polymer/drug ratio (25:1–100:1) and the type of polymer (PLGA 85/15, D,L-PLA). The acetonitrile/acetate buffer method resulted in an acceptable drug recovery of 93.0% ($\pm$ 1.0%, $n = 4$) for PLGA 85/15 (M.W. 87000); the polymer was soluble in this solvent mixture. However, this method was not suitable for polymeric microspheres, which were insoluble in the solvent mixture (e.g. L-PLA) because of incomplete extraction of the peptide. Similar problems were encountered with the third method evaluated, the methanol method.

The W/O/W solvent evaporation method is a complex multiphase process; various preparation variables were investigated in order to identify the critical variables affecting the encapsulation efficiency of the process. The batch-to-batch reproducibility under standard conditions (shown in

Table 1) was good (actual drug loading: 1.55 ± 0.03% w/w; encapsulation efficiency: 84.4 ± 1.2%, $n = 6$). A comparable value was found for D,L-PLA with a molecular weight of 6000 (encapsulation efficiency: 85.2 ± 5.8%, $n = 4$). The encapsulation efficiency was similar (82.2%), when the primary W/O emulsion was prepared by vortex-mixing instead of ultrasonication. Somatostatin-containing emulsions were more physically stable than drug-free emulsions, showing a slower phase separation. The surface activity of the peptide drug apparently promoted the formation of the primary W/O emulsion. Although the encapsulation efficiency was the same for microspheres prepared by vortex-mixing or ultrasonication, the internal structure of the microspheres was different. Microspheres prepared by sonication had a homogeneous matrix with small pores, while large internal voids, probably resulting from larger droplets of the internal aqueous phase, were visible with microspheres prepared by vortex mixing (Fig. 1a and Fig. 1b). The insignificant differences in encapsulation efficiencies between the two batches could have been the result of using only a small volume of internal aqueous phase (the W/O-phase volume ratio was 0.03). Pouring the primary W/O-emulsion from a vial into the external aqueous phase instead of injecting it resulted in lower encapsulation efficiencies (74.7 ± 2.6%, $n = 3$) because of a less efficient emulsification process.

Higher actual drug loadings were obtained by increasing the theoretical drug loading (Fig. 2). The encapsulation efficiencies were similar and greater than 80% in all cases, except for microspheres prepared with a theoretical drug loading of 10% w/w. With the volume of the internal aqueous phase being constant, similar encapsulation efficiencies were expected at these relatively low theoretical loading levels because of a similar volume of the internal aqueous phase coming in contact with the external phase at the droplet interface; a constant drug fraction was therefore lost to the external aqueous phase. Depending on therapeutic requirements, microspheres with varying drug contents could therefore be prepared through variation of the theoretical drug loading.

As expected, increasing the amount of polymer decreased the actual drug loading, but slightly increased the encapsulation efficiency; the theoretical loading also decreased because of a constant amount of drug being used (Fig. 3). Increasing the amount of polymer in the organic phase increased the viscosity of the primary W/O emulsion. This possibly stabilized the internal aqueous phase against coalescence and reduced mixing with and hence drug loss to the external aqueous phase.

The volume of the internal aqueous phase was an important variable for microspheres prepared with methylene chloride as organic solvent (Fig. 4). Increasing the volume fraction of internal aqueous phase (acetate buffer) in the primary W/O-emulsion resulted in lower encapsulation efficiencies. With higher internal volume fractions, increasing proportions of the internal aqueous phase will be on the microsphere surface, thus promoting contact and exchange (drug loss) between the internal and external aqueous phase across the surface of the microsphere droplets. The organic polymer phase acts as a diffusional barrier for the drug between the internal and external aqueous phase; the thickness of this layer decreases with increasing volume of internal aqueous phase. This could also lead to a less stable primary W/O emulsion with larger droplets of the internal aqueous phase being formed. Scanning electron micrographs revealed a highly porous internal structure of microspheres prepared with 0.5 ml acetate buffer (Fig. 1e), compared with a homogenenous, denser structure when 0.1 ml internal aqueous phase was used (Fig. 1a). The volume of the internal aqueous phase had no significant effect on the encapsulation efficiency of microspheres prepared with ethyl acetate. With ethyl acetate, somatostatin acetate precipitated upon the addition of the drug-containing aqueous phase to the polymer solution because of the partial miscibility of water and ethyl acetate. A fine drug dispersion rather than a W/O emulsion (as with methylene chloride) was therefore emulsified into the external aqueous phase. Drug dispersions apparently behaved differently from emulsions, leading to lower encapsulation efficiencies. The higher water solubility of ethyl acetate also affected the internal

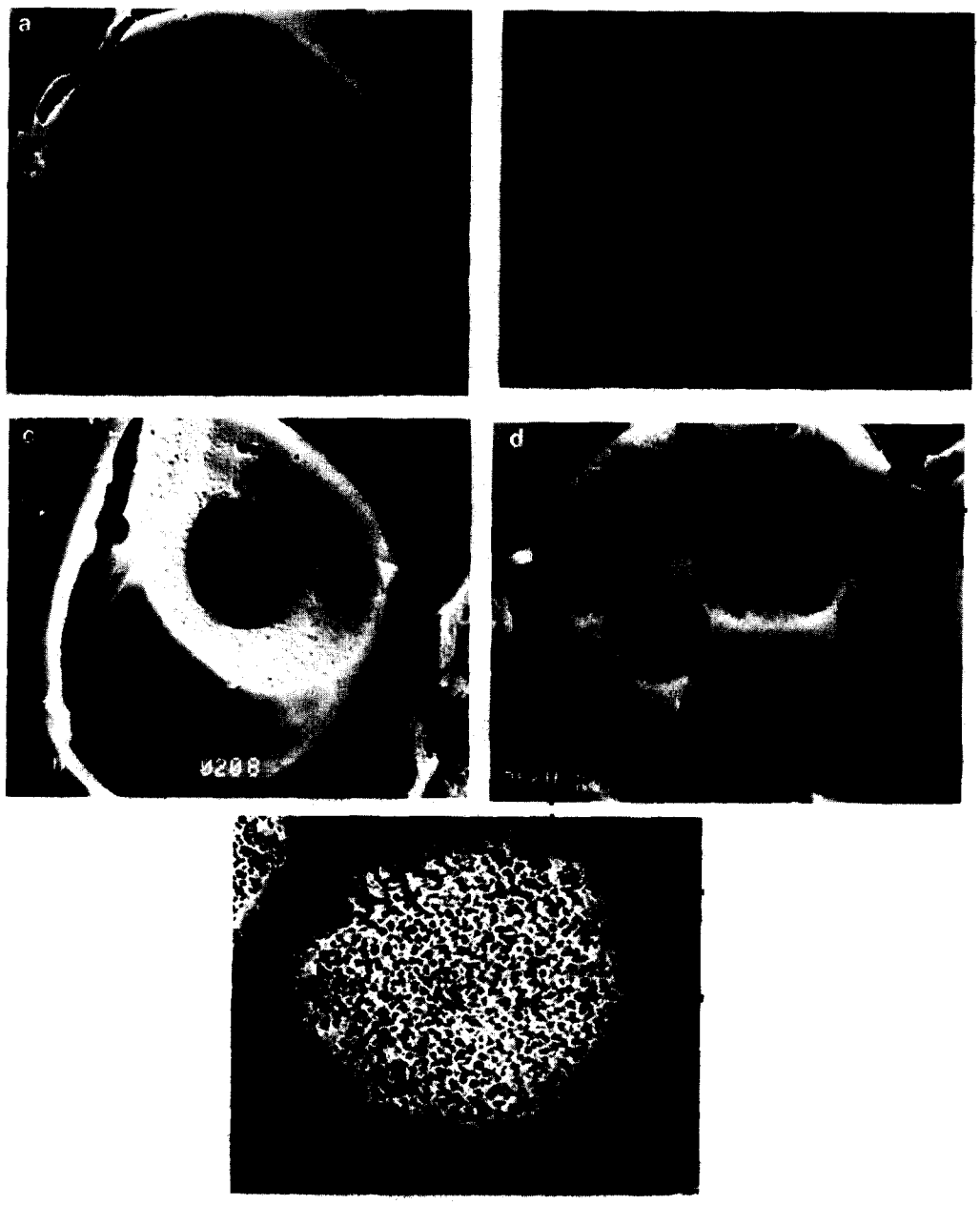

Fig. 1. Scanning electron micrographs of cross-sections of somatostatin acetate containing microspheres. (a) Standard preparation Table 1, drug loading — 1.5% w/w; (b) primary emulsion prepared without sonication; (c) organic solvent — ethyl acetate; (d) organic solvent — methylene chloride/acetone 7:3; (e) volume of the internal aqueous phase — 0.5 ml.

structure of the microspheres (Fig. 1c). After emulsification of the primary W/O emulsion, ethyl acetate diffused rapidly into the external aqueous phase resulting in rapid polymer precipitation at the droplet surface. The microspheres were hollow and non-spherical. The collapsed or deflated ap-

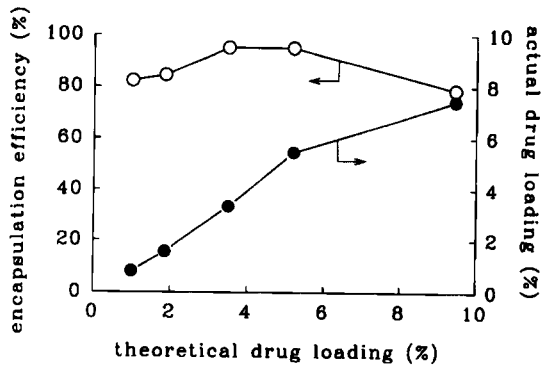

Fig. 2. Effect of the theoretical drug loading on the encapsulation efficiency (○) and the actual drug loading (●).

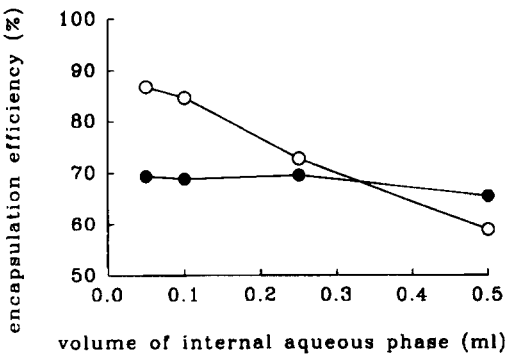

Fig. 4. Effect of the volume of the internal aqueous phase on the encapsulation efficiency. Organic solvent: ○, methylene chloride; ●, ethyl acetate.

pearance of the microspheres was confirmed by optical microscopy in order to exclude the formation of possible artifacts during scanning electron microscopy.

The effect of the volume of organic solvent is shown in Fig. 5. An optimum volume of organic solvent was found for methylene chloride. This reflected the complicated relationship between phase volume ratio, viscosity and stability of the primary emulsion and the rate of polymer precipitation and drug diffusion into the external aqueous phase. An increasing volume of ethyl acetate reduced the encapsulation efficiency. In this case, a high viscosity of the organic phase appeared to be favorable.

Increasing the stirring time reduced the encapsulation efficiency, due to drug partitioning into the external aqueous medium (Fig. 6). This effect was more pronounced with ethyl acetate than with methylene chloride, probably because of the more porous and hollow structure of the microspheres and therefore easier accessibility of the drug by the external aqueous phase (Fig. 1c). Microspheres could not be separated from the aqueous phase in less than 15 min because of agglomeration and fusion of the not fully hardened microspheres when methylene chloride was used as the organic solvent. In order to achieve maximum encapsulation efficiencies, the stirring time should be kept to a minimum. However, this has to be balanced with considerations concerning the residual organic solvent content of the micro-

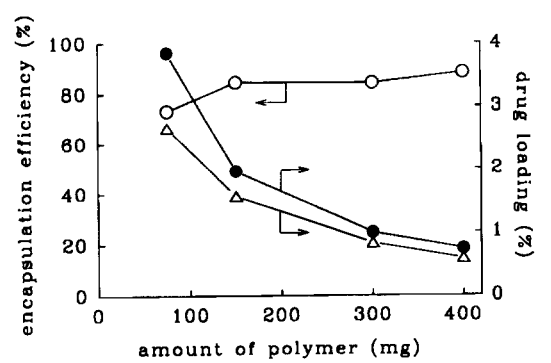

Fig. 3. Effect of the amount of polymer on the encapsulation efficiency (○) and the theoretical (●) and actual drug loading (△).

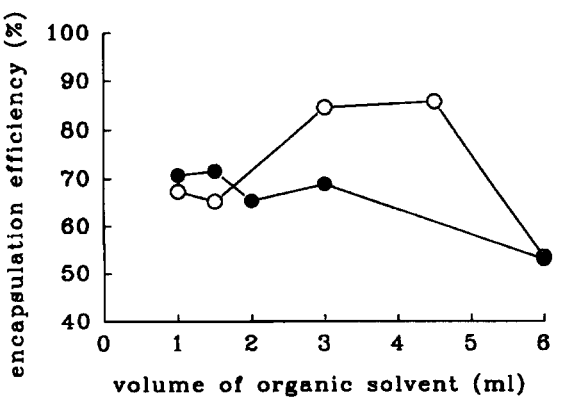

Fig. 5. Effect of the volume of the organic solvent phase on the encpasulation efficiency. Organic solvent: ○, methylene chloride; ●, ethyl acetate.

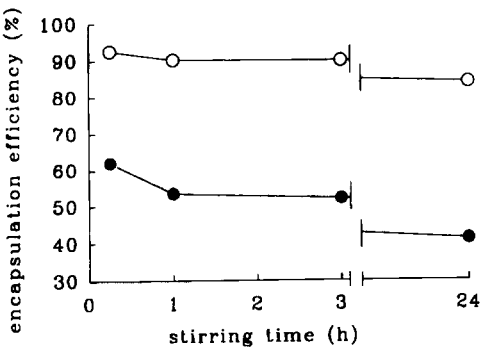

Fig. 6. Effect of the stirring time on the encapsulation efficiency. Organic solvent: ○, methylene chloride; ●, ethyl acetate.

spheres, which will decrease with increasing stirring time. With methylene chloride, only a slight decrease in encapsulation efficiency occurred over a 24 h period, thus allowing for longer stirring times in order to minimize the organic solvent content. Solvent residues in biodegradable microspheres prepared by solvent evaporation methods present a major problem in developing these formulations and for submission to regulatory agencies. Replacing the toxic but widely used methylene chloride with more biocompatible solvents, e.g. ethyl acetate, would therefore be desirable. Microspheres could be prepared with ethyl acetate; however, the encapsulation efficiencies were significantly lower when compared with microspheres prepared with methylene chloride.

The drug release from biodegradable microspheres is governed by a complex interplay of drug diffusion through aqueous pores and the polymer, and by the degradation of the polymer matrix (Washington, 1990). The degradation of polyester homo- or copolymers is primarily affected by the molecular weight, crystallinity and the mole ratio of the monomers (Kissel and Demirdere, 1987). It is interesting to note that the encapsulation efficiency (at these low theoretical drug loadings) was not strongly affected by the type and molecular weight of the biodegradable (co)polymers used. The W/O/W solvent evaporation method resulted in comparable encapsulation efficiencies with various amorphous and crystalline polylactides (D,L-PLA, L-PLA) of different molecular weights and poly(lactide/glycolide) copolymers (PLGA) of varying molar ratios (Table 3). This eliminated the need for adjusting the theoretical drug loading as a function of type and molecular weight of the polymer used, in order to achieve similar actual drug loadings, as long as the molecular weight of the polymer is above a certain threshold. Only the low molecular weight D,L-PLA (M.W. 2000) and PLGA 85/15 (M.W. 6400) showed reduced encapsulation efficiencies, possibly due to the more hydrophilic character of the polymers, the low viscosity of the polymer solutions and the longer stirring time used to solidify these polymers.

Table 3
Effect of polymer type and molecular weight on the encapsulation efficiency

Polymer type	Molecular weight (D)	Encapsulation efficiency (%)
D,L-PLA 100	110 000	93.6
D,L-PLA, Resomer R202	6000	85.2
D,L-PLA, Resomer R104	2000	54.8
L-PLA 100	94 000	85.9
PLGA 85/15	87 000	84.5
PLGA 85/15	6400	44.9
PLGA 50/50	53 000	82.1

The preparation parameters with little or no effect are summarized in Table 4 and Table 5. The particle size of the microspheres had no effect on the encapsulation efficiency, regardless of the type

Table 4
Effect of particle size fraction and organic solvent type on the encapsulation efficiency

Particle size fraction (μm)	Encapsulation efficiency (%)	
	Methylene chloride	Ethyl acetate
45–75	89.5	58.1
75–106	85.1	63.0
106–150	85.5	63.9
150–180	86.0	65.7
180–250	84.8	63.0

Table 5
Preparation parameters with little effect on the encapsulation efficiency

Preparation parameter	Level	Encapsulation efficiency
Concentration of acetone in the organic solvent phase (%v/v)	0	84.5
	10	83.3
	20	80.8
	30	78.5
Volume of the external aqueous phase (ml)	100	81.6
	250	83.4
	500	84.5
	800	85.5
PVA concentration in the external aqueous phase (%w/w)	0	84.6
	0.05	85.7
	0.10	86.4
	0.25	84.5
	1.00	84.9

of solvent. This was in contrast to a previous study, in which the drug loading of microspheres prepared by the same technique (however with much higher actual drug loadings) decreased with decreasing particle size (Chen, 1992). Adding up to 30% v/v acetone to the organic phase or reducing the volume of the external aqueous phase resulted in slightly lower encapsulation efficiencies. The addition of acetone resulted in a porous internal microsphere structure (Fig. 1d). As already observed with ethyl acetate, acetone, being completely water-miscible, resulted in rapid polymer precipitation and hence a porous structure. Although the PVA concentration in the external aqueous phase had no significant effect on the drug encapsulation, the microsphere yield was drastically reduced to 10% without PVA, compared with 85% under standard conditions. A small amount of PVA was therefore needed as a stabilizer to prevent coalescence of the emulsified droplets.

In conclusion, biodegradable somatostatin acetate-containing microspheres could be prepared with high drug encapsulation efficiencies with the W/O/W-solvent evaporation technique. The significance of various preparation parameters with respect to drug loading, encapsulation efficiency and morphology of the microspheres was identified.

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