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Advanced Drug Delivery Reviews 28 (1997) 43–70

advanced drug delivery reviews

One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate

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Abstract

The biodegradable polymers poly(lactic/glycolic acid) (PLGA) and poly(lactic acid) (PLA) were used as wall materials in the preparation of microspheres (msp) containing the LH-RH superagonist leuprorelin (leuprolide) acetate. A novel W/O/W emulsion-solvent evaporation method was devised for the preparation of msp containing this water-soluble peptide. This method achieved high entrapment efficiency and sustained drug release over a long period predominantly due to polymer bioerosion. The msp are fine microcapsules with polycores containing the peptide at a high concentration and are easily injectable through a conventional fine needle. Leuprorelin msp made with PLGA(75/25)-14,000 or PLA-15,000 released the drug in a zero-order fashion, maintained constant serum drug levels and attained persistent objective suppression of the pituitary-gonadal system ('chemical castration') over 1 or 3 months after i.m. or s.c. injection into animals. These results indicate that depot formulations may be potentially useful in the therapy of endocrine diseases in humans. In this paper, studies on the formulation, drug release and pharmacological effects in animals for these leuprorelin depot formulations are reviewed.

Keywords: Leuprorelin (leuprolide) acetate; One- and three-month depot injectable microspheres; Lupron Depot®; Poly(lactic/glycolic acid) (PLGA); Poly(lactic acid) (PLA); Biodegradable polymer; Emulsion-solvent evaporation method; Inhibition of steroidogenesis; Experimental endometriosis; Chemical castration

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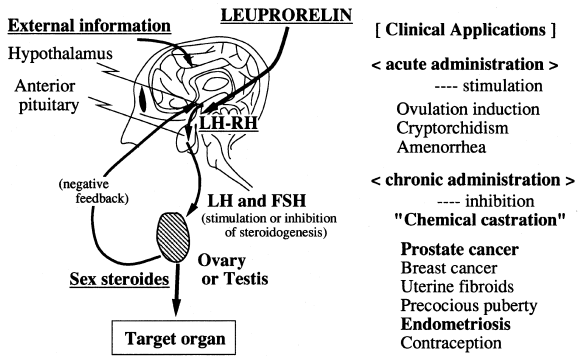
0169-409X/97/$32.00
PII S0169-409X(97)00050-1



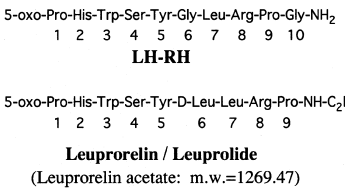


Fig. 1. Amino acid sequence of leuprorelin acetate, its clinical application and schema of its pharmacological effects on the pituitary-gonadal system [31].

1. Introduction

Leuprorelin acetate, Des-Gly^{10}-(D-Leu^{6})-LH-RH ethylamide acetate (Fig. 1), is the first superactive luteinizing hormone-releasing hormone (LH-RH) agonist, and was originally synthesized by Fujino et al. at Takeda Chemical Industries [1]. This analog has about 10 times the biological activity of LH-RH and, at acute doses, stimulates gonadotropin secretion by the pituitary and steroidogenesis in the genital organs. It also induces ovulation. However, when administered chronically at a higher dose, it paradoxically produces antagonistic inhibitory effects on pituitary gonadotropin secretion and testicular or ovarian steroidogenesis ('chemical castration'); these effects, attributable to downregulation of the receptors, are temporary and reversible. These inhibitory effects, which are also observed with several similar LH-RH agonists, have been used in the treatment of hormone-dependent tumors such as prostate [2–4] and breast cancer [5,6], endometriosis [7,8], uterine fibroids [9,10], central precocious puberty [11] and adenomyosis [12] without any serious side effects or need for surgical castration. On the other hand, chronic treatment with LH-RH analogs has the practical disadvantage of requiring long-term daily injections, as they are large water-soluble nona- or deca-peptide molecules which have a short biological half-life after i.v. or s.c. injection and are poorly absorbed from the gastrointestinal tract after oral administration. LH-RH agonists in clinical use are listed with regard to their amino acid sequences, biological potency and biological half-life in Table 1 [13].

Leuprorelin acetate is a very water-soluble nonapeptide with a molecular weight (M_w) of 1269.47. It is fairly stable in water, but is unstable in body fluids and is excreted rapidly. To overcome this disadvantage, we have been attempting to develop oral, rectal, nasal, or vaginal delivery systems for leuprorelin self-administration since 1976. The bioavailabilities (BA) of these formulations were $<0.1\%$, $<1\%$, 1%, and 1–5%, respectively (Fig. 2) [14–21]. The BA for the nasal and vaginal routes was increased by up to 5–10% in humans using absorption promoters. Daily vaginal administration of leuprorelin jelly (500 μg/kg/day) containing 10% citric acid significantly suppressed the growth of hormone-dependent mammary tumors induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) in rats with results comparable to ovariectomy (regression in 82% and disappearance in

Table 1
Amino acid sequence, biological potency and half-life of clinically used LH-RH agonistic analogs [13]

LH-RH analogs (amino acid)	Sites of modification			Biological potency	Activities $T_{1/2}$ (min)
	6th	9th	10th		
Natural LH-RH (10)	Gly	Pro	Gly	1	8
Leuprorelin (9)	D-Leu	Pro-N-Et	—	5–15	(16)
Buserelin (9)	D-Ser-t-Bu	Pro-N-Et	—	25	80
Goserelin (10)	D-Ser-t-Bu	Pro	Aza-Gly-NH_2	75	—
Tryptorelin (10)	Trp-N-Me-Leu	Pro	Gly	100	30
Nafarelin (10)	D-2-napht.-Ala	Pro	Gly	200	144

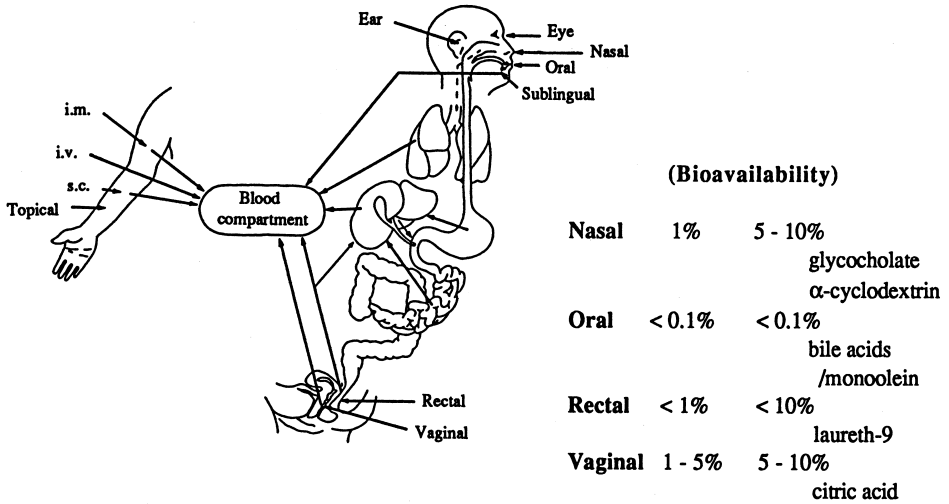


Fig. 2. Administration routes and bioavailability of leuprorelin acetate by various mucosal routes without (left column) or with (right column) an absorption enhancer in humans [21].

53%), and inhibited the development of new tumors [19]. However, absorption through the nasal or vaginal mucosae is influenced by physiological conditions (e.g. ovarian cycle stage or upper respiratory tract infection), and frequent administration over a long period is very inconvenient for patients even by these routes. During our studies on vaginal absorption, we noticed that sustained blood levels of the peptide produced a stronger chemical castration effect than intermittent pulsatile administration, and reasoned that a continuous release injection would produce optimal objective therapeutic results [18]. Based on this finding, Okada and Yashiki focused on the development of a sustained release injection and elaborated a plan for a microsphere-type depot formulation of the peptide using biodegradable polymers. At that time, there was no successful commercial product of this type of formulation in the world and, in Japan, biodegradable polymers such as polylactic acid (PLA) and poly(lactic/glycolic acid) (PLGA) could not easily be obtained. In 1980, we synthesized these polymers in our laboratories and started to investigate preparation methods for microsphere sustained release formulations which released the peptide over 2 weeks or 1 month following a single conventional injection.

Subsequently, a once-monthly injectable depot formulation of leuprorelin was achieved by developing a novel preparation method [22–24]. This was first launched in the US (1989) and is now widely available on the world market [25–33]. A 3-monthly depot was also recently marketed in the US (1995) [34–38]. These depot formulations provide fairly constant release of the peptide over 1 month or 3 months in animals and humans after s.c. or i.m. injection and show sufficiently reliable efficacy for the treatment of patients with hormone-dependent cancers such as advanced prostate cancer [39–51] and mammary tumors [52,53], or other endocrine diseases such as endometriosis [54–60], uterine fibroids [61–70], adenomyosis [12] and precocious puberty [71–75], without severe side-effects [76]. In the treatment of prostate cancer, severe bone pain could be relieved in patients with D_2 stage advanced tumors. Thus, this treatment markedly improves patients' quality of life (QOL) and aids compliance as well as removing the need for painful and troublesome repeated daily injections to achieve persistent chemical castration.

The synthetic biodegradable polymers PLGA and PLA are used as wall materials for polycore microcapsules (mcp) of leuprorelin in these preparations because they have several excellent chemical and biological properties, including good biocompatibility, lack of immunogenicity, easy control of their bioerosion rate by selection of their molecular weight and copolymer composition, lack of metallic catalyst residues, and reproducible msp production

when our method of in-water drying without any crosslinking agents is used. Since these polymers could not easily be obtained in Japan when we started this investigation, they were synthesized in-house and the biocompatibility, erosion and disappearance rate of these polymers was determined for the 1-month depot formulation, injectable microspheres (msp). Suitable msp were achieved by the preparation method originally devised by Okada et al. [22] and Yamamoto et al. [23]. The peptide is released in two phases: initial diffusion from near the surface of the swollen msp (diffusion phase) followed by elution during erosion of the polymer (bioerosion phase). The msp are very fine, about 20–30 μm in diameter, fairly spherical, composed of a solid monolithic matrix with many narrow channels (Fig. 3) [30], contain about 8% of the peptide in the formulation, and are easily injectable using a syringe with a conventional fine needle (22–26 gauge) without local anesthesia. After injection the msp remain at the injection site, show good biocompatibility and disappear about 6 weeks later for the 1-month depot or 18 weeks later for the 3-month depot injection. The msp also show superior qualities in that they contain hardly any residual organic solvent and the possibility of irradiative degradation of the drug and polymer during sterilization is eliminated by preparation under strict aseptic conditions. When using the depot formulation, the effective dose of the peptide is reduced to 1/4 to 1/8 of that needed for repeated parenteral administration of a plain solution due to the promotion of down-regulation by continuous hits on the receptors resulting from sustained peptide levels in the target organ. Recently, a disposable double-chambered syringe [48] in which one chamber is filled with the msp and the other with dispersing vehicle has been developed and is already on the market in the US

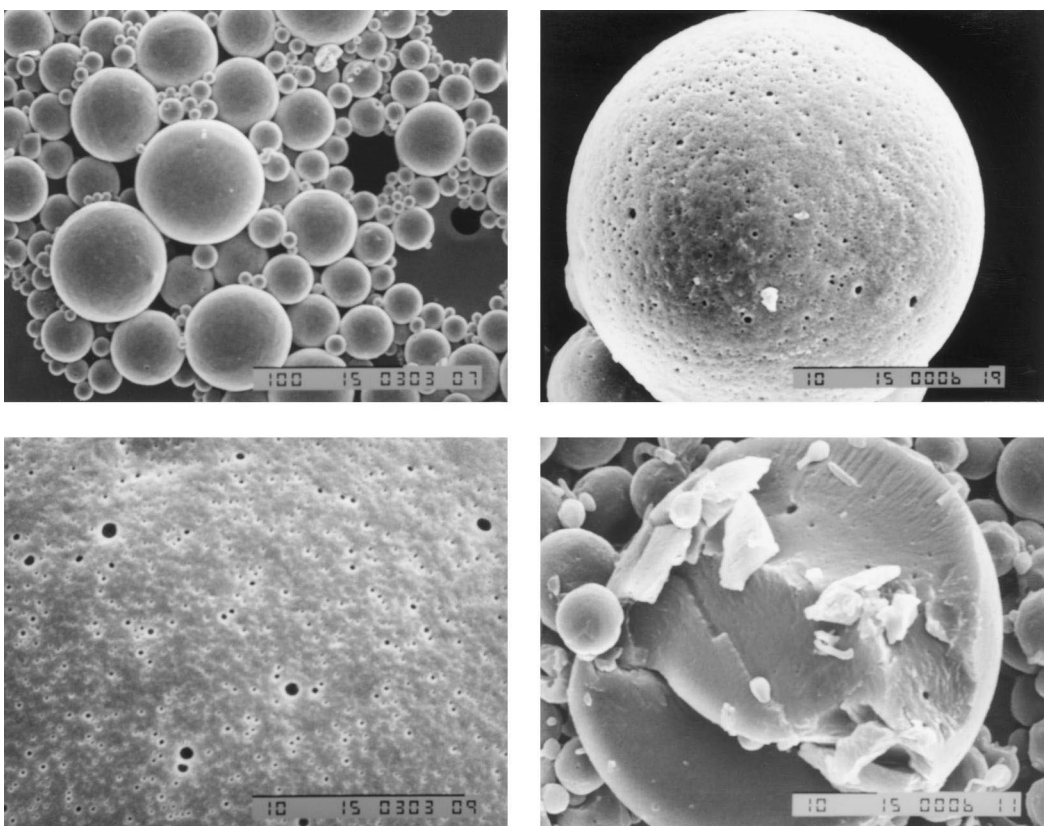


Fig. 3. Scanning electron micrographs of the surface and cross-section of leuprorelin 1-month depot PLGA microspheres (Lupron Depot®) [30].

and EU. The msp are easily dispersed in a single step by pushing in the plunger and shaking. This shortens the preparation time and ensures aseptic reconstitution.

In this paper, 1-month and 3-month release injectable msp of leuprorelin acetate are reviewed with respect to the biocompatibility and biodegradation of the polymers, preparation, drug release, and pharmacological effects in male and female animals.

2. Biodegradation and biocompatibility

We decided on PLA and PLGA as wall materials for the msp because these polymers have already been utilized as biodegradable surgical suture materials and are known to be biocompatible. To determine the most suitable polymer for the 1-month depot injection, PLA (M_w 6800–73 000) and PLGA (copolymer ratio 90/10 to 25/75, M_w 10 000–20 000) were synthesized and their bioerosion rates after implantation in rats and leuprorelin release profiles from the msp were determined [77].

The low M_w PLA and PLGA used in this study (weight average M_w $<30\,000$) were synthesized by direct polycondensation using the resin-catalyzing method reported by Nevin [78]. The strong anion-exchange resin used to catalyze the polymerization of the DL-lactic and glycolic acid monomers is excellent, as it is easily removed by filtration once the reaction is complete. The polymers used in the products were synthesized by direct condensation without catalysts, a method developed by Wako Pure Chemical Industries in collaboration with our laboratories. High M_w PLA was synthesized from the cyclic dimer, lactide, by the ring-opening method using diethyl zinc as a catalyst, as reported by Woodland et al. [79]. The M_w was determined by gel-permeation chromatography (GPC), and the lactic/glycolic (LA/GA) copolymer ratio of PLGA was obtained from the integrated signal ratio between the methyl moiety of lactic acid and the methylene moiety of glycolic acid in the nuclear magnetic resonance (NMR) spectrum. Lactic acid contains an asymmetric carbon atom and has two optical isomers. Therefore, it produces L-, D- and DL-lactic acid polymers. The L- and D-polymers have a crystalline form, but the DL-polymers are suitable for our depot formulations as they are amorphous and do not lead to any morphological changes in the msp during storage and distribution.

Polymer plates, 1 mm thick, were prepared by a hot-press method and cut into square pieces measuring 10×10 mm for s.c. implantation into the backs of rats. The polymer was periodically removed to determine any changes in the remaining weight and M_w after drying. As shown in Fig. 4, the typical pattern of weight loss of the polymer plates was biphasic, with an initial lag time followed by a period during which the weight fell at a first-order rate. Erosion of the polymers was enhanced by a decrease in M_w, while the lag time and half-life increased linearly with increased M_w. An increase in the proportion of copolymerized glycolic acid in PLGA resulted in rapid erosion upto an LA/GA ratio of 50/50, in line with the result reported by Miller et al. [80]. The M_w of all polymers began to decrease gradually soon after implantation, and the lag time became shorter. After s.c. implantation the polymers become hydrated, swell relatively rapidly, and gradually degrade throughout their matrix (bulk erosion). When water-soluble oligomers (M_w 400–600) are produced, a decrease in weight occurs as these oligomers are eluted from the plate. The period before elution starts corresponds to the lag time. However, a very important consideration for practical use was that the lag times for msp composed of low M_w PLA and PLGA were relatively short because water and oligomers were able to diffuse easily through the small polymer matrix. During this experiment, an interesting phenomenon became evident: the M_w of the outer phase of the polymer plate, which was attached to the tissue, was higher than

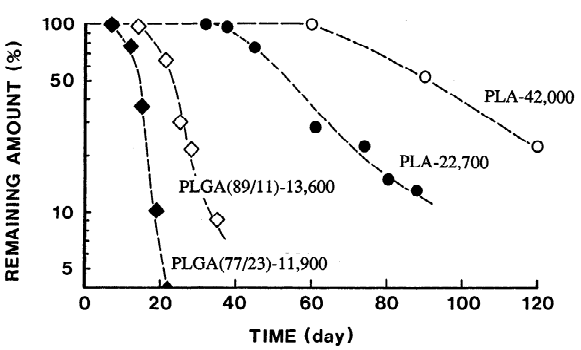


Fig. 4. Biodegradation of PLGA and PLA after s.c. implantation in a square plate in rats (mean weight of the plate remaining at the implantation site, $n=3$).

that of the inner phase. The outer phase was always solid but the inner phase was sometimes semisolid after erosion. This phenomenon was assumed to be due to self-catalysis of degradation by the oligomers produced during the process, which were not easily exuded from the inner phase. This heterogeneous degradation in PLA plates has also been demonstrated in phosphate buffer by Vert et al. [81]. Since oligomers in the polymer enhance polymer hydrolysis and increase the initial burst of drug release, the polymer is treated to remove oligomers before use [82].

The polymer used for a 1-month depot injection should disappear from the injection site within 2 months. Therefore, it is clear that the polymer should be a relatively small one: a M_w of 6000 for PLA and 12 000–14 000 for PLGA is optimal. An increase in the glycolic acid content of PLGA reduces its solubility in dichloromethane (DCM), and this makes it difficult to prepare msp by the in-water drying method. Local irritation has often been caused by plates containing low M_w PLA (e.g. 6000), depending on the erosion rate (i.e. the concentration of the monomer or oligomers at the implantation site) and the shape of the implant. On the basis of these results and release studies following preparation of the msp, PLGA (75/25) was finally selected because it shows less variation in its erosion rate after a change in the copolymer ratio [83], and has a higher glass transition temperature (T_g) accompanied by a higher M_w in comparison with PLA providing the same erosion rate. The T_g of PLA and PLGA is about 30–60°C and is represented by the following equation:

$$T_g = T_g^0 - K/M_n,$$

where T_g^0 (60.1°C for PLA) is the limiting T_g of a material of infinite M_w, M_n is the number average M_w and K (37.1×10^4°C for PLA) is a constant for the polymer [84]. For the 3-month depot, a more slowly degradable PLA was selected and was shown to be more biocompatible. Following the erosion of PLGA msp, the LA/GA ratio of the polymer increased from 75/25 to about 80/20 4 weeks after s.c. injection, as it did after incubation in pH 7.0 buffer. This was attributed to the bonding of LA-LA being more stable than that of LA-GA or GA-GA [77,85].

The biocompatibilities of PLA and PLGA were originally demonstrated using sutures by Cutrigh et al. [86] and Crag et al. [87]. Sutures composed of these polymers induced a mild local inflammatory reaction when implanted into rats with infiltration of macrophages (Mϕ), giant cells and fibroblasts. They were also surrounded by a thin layer of connective tissue until the sutures were absorbed. Anderson and Marchant [88] and Yamaguchi and Anderson [89,90] described the general events that occur following implantation or injection of biomaterial devices. A blood clot forms around the device and permeability changes in the adjacent vasculature resulting in the exudation of plasma components and mediators, followed by migration of neutrophils and a marked increase in polymorphonuclear leukocytes (PMN). This is a typical early phase inflammatory reaction. The major role of the neutrophil is to phagocytose microorganisms. It then dies and releases its cytoplasmic and granular components, mediating the subsequent inflammatory response. Following the depletion of PMN, monocytes migrate to the injection site and undergo differentiation into Mϕ, which are principally responsible for normal wound healing. Fibroblast recruitment with collagen deposition and formation of a fibrous tissue capsule and capillary proliferation then occurs as a chronic response. In general, the tissue response to msp involves very localized or focal granulomatous inflammation consisting of Mϕ, foreign body giant cells and capillary infiltration. Collagen deposition with fibrous capsule formation is minimal, and there is a lack of necrosis in the adjacent tissues. The msp implantation sites are gradually replaced by collagenous tissue during degradation. At the msp/tissue interface, the predominant cell type changes from the Mϕ to the foreign body giant cell; the Mϕ resumes predominance later. These changes in cell type are thought to be caused by the decreasing sizes of the msp left to scavenge.

Visscher et al. [91–93] also described in detail the tissue response after i.m. injection of PLGA or PLA msp (30 μm) containing lysine-8-vasopressin, together with the morphologic changes occurring in the msp, based on histological observations. In the case of a device with a 1-month lifespan, a minimal inflammatory reaction characterized by infiltration of lymphocytes, plasma cells and histocytes, together with acute myositis, was observed soon after injection. By day 4 post-injection, a minimal subacute

inflammatory response had occurred, and the msp were enmeshed in a thin connective tissue capsule consisting of extracellular collagen and fibrin, with accumulation of Mϕ, giant cells, neutrophils, fibroblasts, lymphocytes, and plasma cells. Between days 11 and 42, the msp were surrounded by chronic inflammatory cells with large prominent Mϕ, foreign body giant cells and fibroblasts. On days 30 and 42, infiltration of giant cells was seen within some caviated msp; however, there was no indication that phagocytosis by Mϕ or giant cells was important in the biodegradation process. By day 63, complete erosion and breakdown of the msp had occurred and the minimal chronic cell response had almost completely resolved. These studies suggest that the tissue response does not play a major role in controlling the degradation rate of polymers, but it is not clear at present whether these responses influence their degradation and drug release even, in smaller msp.

PLGA(50/50)-122,000 microspheres (22 μm) prepared by the solvent evaporation method after sterilization by γ-irradiation were injected into deep areas of the rat brain, such as the striatum, using stereotaxis to bypass the blood-brain barrier, and the resulting tissue response was examined [94]. Nonspecific astrocytic proliferation and macrophagous-microglia cell reaction, like that typically found following physical damage to the central nervous systems (CNS), was observed. Some foreign-body giant cells were detected between days 3 and 7; these decreased between weeks 2 and 4, persisting only locally. Some activated microglial cells and Mϕ expressed class II MHC antigen, but no T-lymphocytes were observed at the implantation site at any time, and no fibroblasts were detected. PLGA msp therefore do not appear to produce neuronal toxicity or necrosis in brain tissue. This device is thus concluded to be biocompatible with the brain.

The biocompatibilities of the 1-month and 3-month depot formulations of leuprorelin were assessed in rats after s.c. injection and in rabbits after i.m. injection (unpublished data). Periodic histological examination of the injection site revealed a well-tolerated minimal inflammatory reaction with infiltration of several types of inflammatory response cells as described above. This was surrounded by a thin connective tissue capsule, on which fine capillaries developed. The capsule had the appearance of a living organ, producing and secreting the peptide continuously into the systemic circulation. An intramuscular irritation test of the msp in rabbits confirmed their good biocompatibility with no inflammation and mild angiogenesis around the connective tissue and gradual disappearance of the msp following bioerosion. In this test, the activity of serum creatine phosphokinase, a marker of chronic inflammation, was slightly elevated 1 day after injection (normal: 300 mU/ml, msp: 700 mU/ml, vehicle: 550 mU/ml, 0.1 N acetic acid: 1250 mU/ml) but returned to the normal level after 2 weeks.

3. Preparation procedures for microspheres

3.1. One-month depot formulation

There are several methods of microencapsulating a water-soluble compound [33]. The major methods can be divided into two categories: the emulsion solvent evaporation method and the phase separation method [95–97]. We initially selected the emulsion method, based on a novel W/O/W emulsion technique (in-water drying method) for water-soluble substances originally devised by Okada et al. [22] and improved by Yamamoto et al. [23]. This was selected because the procedure is simple, the materials required for parenterals are easily available and the few organic solvents used have a low boiling point, which overcomes the problems associated with residual solvents. The bench scale preparation procedure is illustrated in Fig. 5. The peptide (450 mg) and gelatin (80 mg) are dissolved in a small amount (0.5 ml) of distilled water (W_1) at about 60°C. PLGA (4 g), dissolved in 5 ml dichloromethane (DCM) (O), is mixed with this solution and agitated vigorously with a homogenizer. This W_1/O emulsion is cooled to about 15–18°C to increase the viscosity of the inner water phase and the emulsion itself, then poured into 1250 ml chilled 0.25% polyvinyl alcohol (PVA) (W_2) solution through a long, narrow nozzle with stirring using a turbine-shaped mixer at 6000 rev./min. The resulting $W_1/O/W_2$ emulsion is stirred gently for 3 h to remove the organic solvent, and semi-dry msp are obtained. These msp are sieved through 74-μm apertures to remove larger particles, collected by centrifugation after washing twice with water, redispersed in mannitol solution and lyophilized to complete the removal of the

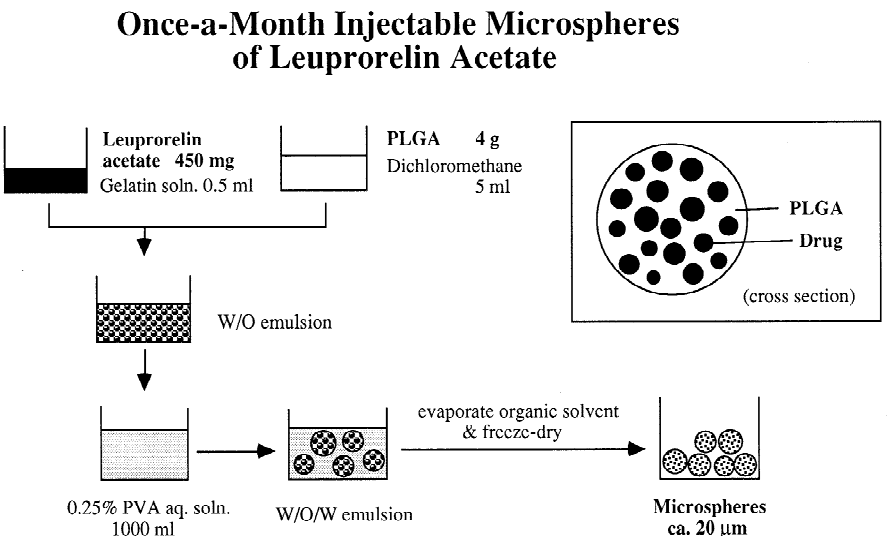


Fig. 5. Preparation procedure for 1-month depot PLGA microspheres of leuprorelin acetate using a W/O/W emulsion-solvent evaporation method [33].

organic solvent and water. In this procedure, only one volatile organic solvent (DCM) is used and the residue is reduced to below 100 ppm by well-programmed heating during the lyophilization process. The limit for this solvent in the US Pharmacopeia (section 467, Organic Volatile Impurities, USP 23, 1995, p. 1746) is 500 ppm. When using the solvent evaporation method, formation of a stable W/O/W emulsion is required. After several experiments using non-ionic surfactants with differing amphiphilic balances, we found that a stable W/O emulsion could easily be obtained by controlling the temperature in addition to the concentration and volume of the peptide and polymer solution because an aqueous solution of the peptide, PLGA and PLA has surface-active properties when dissolved in a polar solvent such as DCM and the resulting W/O emulsion is viscous. If surfactants had to be used in the inner water or oil phases, their elimination from the msp would be extremely difficult. Furthermore, the incorporation of PVA in the outer water phase as a colloidal protector, as used by Beck et al. [98], allowed the formation of a very stable W/O/W emulsion and played an important role not only in dispersing the oil particles but also in preventing aggregation of the semi-dry msp due to increased viscosity and water wettability. However, in general, water-soluble substances, especially those with a low molecular weight, are easily released to the outer water phase even after a stable W/O/W emulsion has been formed during solvent evaporation over several hours. Bodmeier and McGinity increased the trapping efficiency for a water-soluble drug by saturating the outer water phase with the drug [99]. This method could not be utilized for leuprorelin because of its exceedingly high solubility. A high trapping efficiency could, however, be achieved by increasing the viscosity of the inner water phase (by adding gelatin to the water phase and lowering the temperature) and the stability of the W/O emulsion (by increasing the concentrations of the drug and polymer). Adding gelatin and lowering the temperature of the W/O emulsion produced a dramatic increase in the trapping efficiency of a water-soluble peptide from 6–7% to 70% using the original in-water drying method [22]. It has been speculated that the high trapping efficiency is produced by the formation of a rigid matrix structure due to rearrangement of the polymer molecules surrounding the drug core. This probably results from a strong interaction between the cationic drug and the anionic polymer [32]. Subsequently, the trapping efficiency has been further improved by increasing the concentrations of the drug and polymer solutions and decreasing their volumes [23]. In scale-up studies, further strict optimization of the formulation using PLGA(75/25)-

14,000 and improvement of the preparation apparatus has allowed consistent attainment of entrapment percentages of more than 95%. When the viscosity of the W/O emulsion became too high (more than 8000–12 000 cp), non-spherical msp resembling strings were observed due to insufficient shear with the homogenizer; however, their release profile was no different from that of spherical msp. When different drugs and drug loads are used, or the type of polymer is changed, the volumes of water and organic solvent and the temperature of the W/O emulsion should also be changed in order to obtain highly entrapped msp with a spherical, smooth surface and constant sustained release.

The msp prepared by our emulsion method are polycore mcp in which many fine drug cores are dispersed throughout the interior of the polymer matrix (Fig. 5). If the drug core becomes large as a result of drug crystallization, insufficient homogenization of the W/O emulsion or excess drug loading, the trapping efficiency decreases and the initial drug release burst increases. Mannitol, added to distilled water to redisperse the semi-dry msp before the lyophilization process, is a key compound in these depot formulations because it prevents aggregation of the msp during the lyophilization process and during distribution and storage of the commercial product (patent applied for). The drug content and release profile of the 1-month depot msp preparation of leuprorelin containing PLGA(75/25)-14,000 is scarcely changed after storage for 3 years at room temperature (RT) or for 6 months at 40°C, because the peptide is chemically very stable, the msp are maintained at extremely low humidity levels in the vial and are prevented from aggregating by mannitol, and the T_g of the msp rises with the peptide load due to an interaction between the peptide and the polymer.

For factory production, all procedures are carried out under well-controlled aseptic conditions, all the materials (drug/gelatin, polymer and mannitol solutions) being sterilized by filtration before microencapsulation instead of the final product being sterilized by γ-irradiation. This is because γ-irradiation causes marked degradation of the drug and polymer and determination of the toxicity and bioactivity of all the degradation products would be impossible. The molecular weight of the polymer would also vary according to the irradiation conditions, and the polymer would produce oligomers that might induce a 'tunnel effect' and promote accelerated polymer degradation by self catalysis. An increase in initial peptide loss and shortening of the release period due to the presence of oligomers created by irradiation were reported by Ruiz and Benoit for tryptorelin msp containing PLGA(50/50) [100]. Moreover, the presence of oligomers also affected the phase separation of the PLGA during the microencapsulation process; it was barely possible to isolate well-individualized msp. In our products, the polymers (PLGA and PLA) are washed well with water in a solution of DCM to remove water soluble oligomers before being solidified and milled.

The msp are packed into the vial or dual chamber disposable syringe by an automatic filling machine following automatic checking on a balance under a laminar flow of dry aseptic air (class 100). The containers are then sealed. The msp are easily dispersed just before injection in the reconstitution vehicle, which is composed of carboxymethylcellulose sodium (0.5%), mannitol (5%) and Tween 80 (0.1%) in water for injection (1 or 2 ml for a 7.5 mg dose of the drug). The dispersibility of the msp is influenced by many factors such as the charge on the drug in the msp, the pH, tonicity and viscosity of the vehicle, and the kind and concentration of other components (electrolytes and surfactants) of the vehicle. The vehicle composition also affects the syringeability, which is regulated mainly by the size and shape of the msp and concentration of the msp suspension; the details of these phenomena are rarely reported for other formulations.

3.2. Three-month depot formulation

Three-month release microspheres of leuprorelin have also been prepared by the W/O/W emulsion solvent evaporation method used for the 1-month depot formulation with minor modifications to the volumes of solvents and the elimination of gelatin from the inner drug solution as reported by Okada et al. [36]. In the case of PLA msp loaded with 12% drug, 550 mg drug dissolved in 1 ml distilled water (W_1) and 4 g PLA dissolved in 7.5 ml DCM (O) are mixed and agitated vigorously with a homogenizer to form a W/O emulsion. This emulsion is poured into 1000 ml 0.25% PVA solution (W_2) with stirring. The resulting $W_1/O/W_2$ emulsion is stirred gently for 3 h

to evaporate the organic solvent and obtain the msp. The semi-dry msp are sieved through 74-μm apertures to remove large particles then centrifuged at 1000 rev./min for 5 min to remove very fine particles and isolate them from the outer water layer. The resulting msp are washed with water by repeated dispersion and centrifugation, and finally redispersed in mannitol solution and lyophilized. Because the viscosities of the polymer solution and W/O emulsion are increased, increased volumes of the inner water phase and DCM are required, and gelatin can be eliminated from the inner drug water phase. The msp are fairly spherical particles with a mean diameter of about 20 μm and have many small pores on their surface, similar to those in the 1-month depot preparation. These small surface pores are presumably produced by the evaporation of DCM during in-water-drying and lyophilization. The trapping efficiency is very good, $>$95% for drug loads of between 5 and 18%. The dispersing vehicle (1.5 ml for a 22.5 mg dose of the drug) is the same as that for the 1-month depot product.

The stability of peptides and proteins entrapped in msp containing PLA and PLGA is one of the most important considerations for long-term delivery systems. Further attention needs to be paid to possible sources of instability during the process of msp preparation; for example, interaction with organic solvents, mechanical stress during emulsification, and dry heating may cause degradation. Numerous studies of rapid degradation or degeneration of peptides and proteins during preparation processes and drug release have been reported, for example, porcine somatotropin [101], atriopeptin III (rat atrial natriuretic factor) [102], insulin [103,104], trypsin [103], superoxide dismutase (SOD) [104], tetanus toxoid [105], recombinant human growth hormone (rhGH) [106,107], interferons (rIF-γ) [107], nerve growth factor (NGF) [108] and interleukins (IL-1α) [109]. The instability of peptides and proteins during release processes is sometimes catalyzed by the polymers used in the formulation and accelerated by shaking. The instability of SOD due to the use of DCM during preparation was reduced by using the reversed micelle solvent evaporation method followed by solubilization in DCM with sucrose esters of fatty acids as a surfactant [104]. Fairly effective adjuvants for stabilization have also been discovered, including gelatin and human serum albumin (HSA) for tetanus toxoid [105], trehalose and mannitol for rhGH and rIF-γ [107], dextran (M_w 70 000) for NGF [108], and bovine serum albumin (BSA) [109].

4. Drug release, pharmacokinetics in animals and formulation studies

4.1. One-month depot formulation

The in vitro and in vivo release rates of drugs from msp prepared using several PLA and PLGA polymers have been determined [24,77]. For the in vitro release study, the msp were dispersed in 1/30 M phosphate buffer (pH 7.0) containing 0.02% Tween 80 at 37°C under rotation and were collected at appropriate intervals by filtration through a 0.8 μm Millipore filter. The drug in the msp was measured by high-performance liquid chromatography (HPLC) following extraction with pH 6.0 phosphate buffer after dissolution in DCM. The release rate was affected by the composition of the buffer and its concentration as well as the pH, tonicity and temperature of the buffer solution, but less by shaking. The above conditions were selected for the in vitro release test after studying these factors. In vivo release was determined in rats after s.c. or i.m. injection of the msp. The amount of peptide remaining in msp excised from the injection site was also measured by HPLC after homogenization and extraction [26]. Because most of the msp were well localized at the injection site and were encapsulated by a thin layer of connective tissue, they could be easily excised together with the connective tissue and analyzed quantitatively. Fortunately, the acidic microenvironment of the msp due to production of water-soluble oligomers following bulk erosion means that leuprorelin is rarely degraded within the msp because the peptide is at its most stable at pH 4.0 (unpublished data). The microencapsulated peptide appears to be protected from enzyme attack in the tissue since the BA is not less than that estimated from the release rate and total clearance of the drug [26], and similar release patterns have been observed in in vivo and in vitro release tests [77].

The release rate from msp depends predominantly on the polymer erosion rate; msp made with relatively stable polymers such as PLA-22,000, PLA-

12,000 and PLGA(90/10)-21,000 show slow release: only 20–30% at 4 weeks after injection. In contrast, msp prepared with PLA-6000 and PLGA(75/25)-14,500 gradually release the peptide over 4 weeks, the latter releasing 80% of the peptide by 4 weeks after injection. In a preliminary study of changes in the estrous cycle of female rats after injection of msp made with several kinds of polymers, the cycle was arrested at diestrus due to continuous stimulation following sustained release of the drug, revealing for the first time the possibility of long-lasting release associated with erosion of the polymer used in this system [22]. Subsequently, studies of the amount of drug remaining at the injection site and blood drug levels have confirmed the continuous release of the drug from the msp at a near zero-order rate. In msp made with PLA-22,000, drug release was very slow and a time lag was observed over the first few days [83]. Hydrophilic substances such as monooleic glyceride were therefore added to the msp to form aqueous channels and enhance drug diffusion. However, although initial release was enhanced, release during the erosion phase was not promoted because these additives did not promote erosion of the polymer. These results indicated that continuous release for 1 month could not be obtained by selecting other polymers, and that reproducible release control could only be achieved by adapting a suitable polymer and regulating the release rate by controlling its erosion. Thus, we decided to use PLGA(75/25)-14,000 as the polymer for the 1-month depot formulation.

The release profiles for the one-month depot formulation, expressed as the amount of the peptide remaining at the injection site in rats after s.c. injection of three different doses, are shown in Fig. 6 [110]. The msp provided pseudo-zero-order release at a rate of 2.5% of the dose/day for 1 month after a small initial burst release of less than 20% of the total dose; the release profiles were similar for each dose. Although the release profiles appeared to be almost linear, they consisted of two exponential curves due to diffusion from near the surface (initial diffusion phase (a)) and release coinciding with erosion of the polymer (bioerosion phase (b)), both of which progress at a first-order rate [28]. These two release phases were well coordinated over 1–2 weeks and provided apparent zero-order release for 4 weeks. The bioerosion phase is very important in

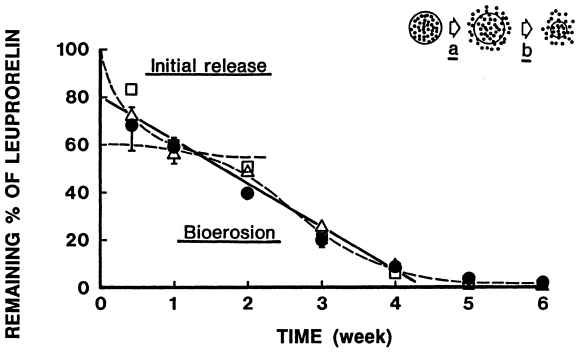


Fig. 6. In vivo release profiles of leuprorelin 1-month depot microspheres in rats after s.c. injection [31] (dose = 0.9 (●), 3 (△), 9 (□) mg/rat, mean ± S.E., $n = 5$).

limiting the releasing period. For the first week, the change in weight of the polymer is small (Fig. 4) and corresponds to the lag time, and elution of the oligomers produced during polymer erosion causes release of the drug from the inner phase of the msp. Water penetrates throughout the msp within 1 day to induce the initial phase release, contributing to the bulk erosion of the polymer and gradual release of peptide. The polymer that diffused out with the drug had almost disappeared by 6 weeks after injection. Similar release profiles were obtained in rats after i.m. and s.c. injection of the msp [26]. Since the erosion of PLGA at the injection site is caused primarily by hydrolysis with water but not enzymes, any differences due to the injection site selected would be expected to influence the release profile only slightly. The negligible effect of enzymes on the bioerosion of PLGA is also proved by the lack of species differences and the high correlation between the in vivo and in vitro release pattern.

The effect of the peptide load (up to 30%) on release has also been determined [24]. Loading up to 14% did not influence the trapping efficiency or the linear release of the msp. However, increasing the load to more than 20% caused obvious elevation of the initial release, most likely due to an increase in the number of aqueous channels which would connect the dispersed drug cores. The drug load in the final formulation was set at 11%.

The particle size of the msp is also a very important factor which influences the drug release profile as well as dispersibility and syringeability during injection. The in vivo drug release of four

kinds of msp with several particle sizes (1–120 μm) was determined in rats. The smaller msp tended to show an increase in initial release, but the release rate was not proportional to the surface area and the influence of particle size was unexpectedly very small. This might be explained by the fact that these msp contain many small pores about 1 μm in diameter, produced by evaporation of the organic solvent, through which water can easily penetrate, and the effective surface area available for drug release, including these inner pores, is much greater than the outer surface of the msp. Furthermore, Visschel et al. [111] reported that the in vitro erosion rate of the polymer, which predominantly controls the release duration, was not affected by mean particle diameters between 30–130 μm. In our formulation, larger (more than about 100 μm) and smaller (less than a few μm) msp are eliminated by sieving through 74 μm apertures and by centrifugation when collecting the msp following in-water drying, respectively. The particle size and distribution of the product is also well controlled by strict regulation of the conditions for emulsification of the W/O/W emulsion and the addition of mannitol.

From the standpoint of practical use, the possibility of dose dumping caused by bathing or rubbing the injection site was investigated (unpublished data). The effect of bathing was determined from the in vitro release rate after increasing the incubation temperature to 42°C for 35 min (5 min bathing daily for 7 days) or 210 min (30 min bathing daily for 7 days) per week. The amount of drug remaining in the msp was measured 1 day after rubbing for 3 min at 1 or 2 weeks after s.c. injection in the rat. No effects on the release profiles were observed in either experiment, thus these forms of stimulation were confirmed to cause negligible dose dumping from the msp.

Fig. 7 shows the gradual morphological changes in the msp during the in vitro release test as observed by scanning electron microscopy (SEM) [33]. One day after dispersal in phosphate buffer at 37°C, the surface of the msp was still smooth with many small pores, while slight surface erosion was observed

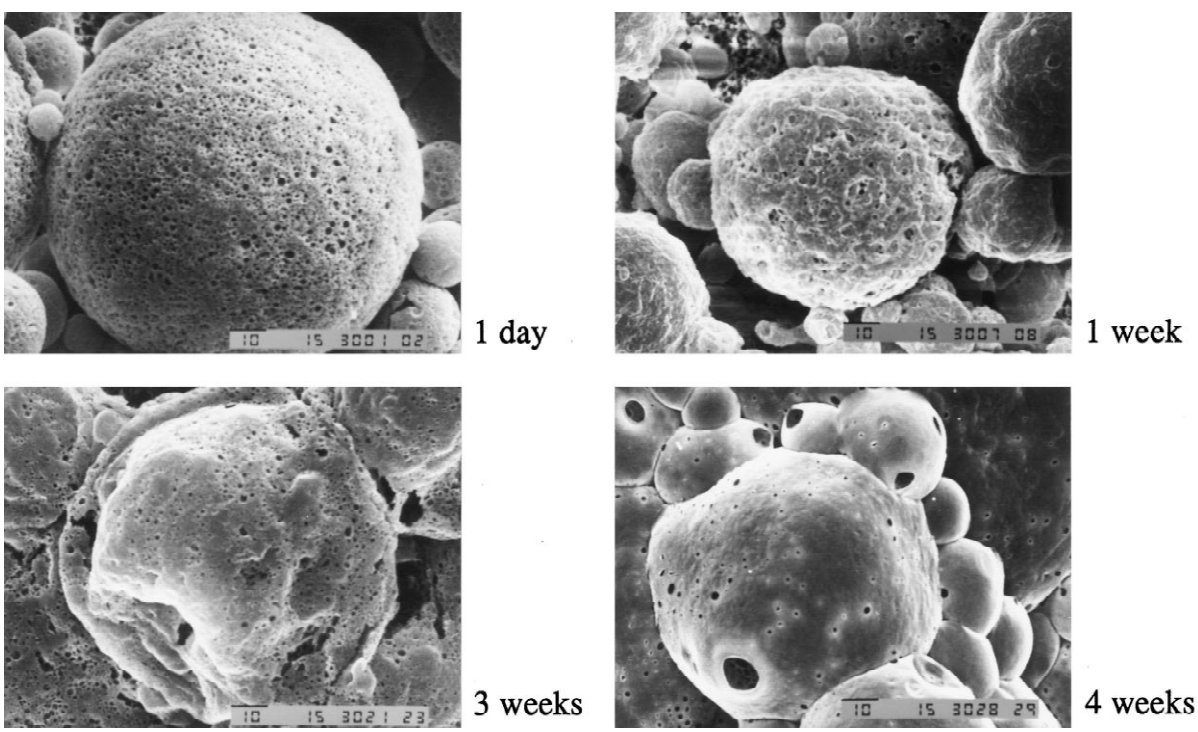


Fig. 7. In vitro morphological change in leuprorelin 1-month depot microspheres immersed in pH 7.0 phosphate buffer containing 0.02% Tween 80 at 37°C [33].

after 1 week of incubation. After 3 weeks, shrinkage and softening of the msp with progressive erosion, which induced deformation on filtration, were evident. Four weeks later, advanced erosion was apparent both on the surface and inside the msp and the surface became smoother. However, the msp were seldom broken apart over this 4-week period and gradually decreased in size and number following bulk erosion, indicating gradual release of the drug with polymer erosion. In vivo, the eroded microspheres aggregated to some extent after being surrounded by a thin collagen capsule. With this depot formulation, the in vitro release was slightly delayed, especially during the terminal phase, but correlated very well with the in vivo release. The in vitro drug release profiles were appreciably influenced by several characteristics of the medium, such as the pH [112,113], ion species and capacity of the buffer, ion strength [114,115], tonicity [116], surfactant, temperature and presence of esterase enzymes [112]. Setting up the system for the in vitro release test is therefore very complicated and must be done carefully. A small volume of medium might cause slow release of the drug due to saturation by the drug or PLGA oligomers.

The drug release profiles of the final formulation were determined by evaluation of serum drug levels using a radioimmunoassay (RIA) system [26,117] after injection of the msp. Sustained serum levels of the peptide were maintained over 4 weeks in rats and dogs after a single s.c. or i.m. injection [26,27,110]. Fig. 8 shows sustained serum leuprorelin levels in rats after three s.c. injections of the msp repeated at 4-week intervals, and the resulting persistent suppression of serum testosterone levels [29,30]. The plateau serum drug level (C_{ss}) was calculated by assuming a constant infusion of the drug using the equation $C_{ss}=k/Cl_{tot}$, where Cl_{tot} (Dose/AUC) is the total body clearance of the peptide after a bolus injection of the solution and k is the release rate calculated from the amount of msp remaining at the injection site. AUC represents the area under the serum peptide level vs. time curve.

Cl_{tot} after s.c. injection of the peptide solution decreased in the order rat>dog>human (7.8:2:1) [29]. The C_{ss} calculated for rats and dogs agreed well with the experimental value, indicating low levels of chemical degradation and metabolism while the peptide was contained in the msp [27,110].

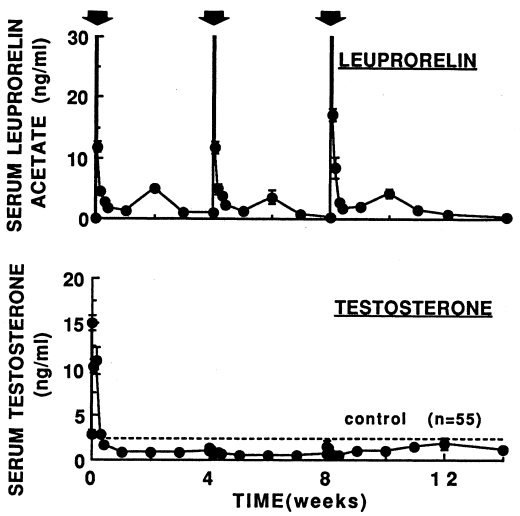


Fig. 8. Serum levels of leuprorelin acetate and testosterone in rats after three repeated s.c. injections of leuprorelin 1-month depot microspheres at 4-week intervals [29,30] (dose = 100 μg/kg/day, mean ± S.E., $n=5$).

Serum drug levels in rats after injection of three different doses (100–500 μg/kg/day) were well sustained for over 4 weeks, and the plateau levels and AUCs were proportional to the dose [29]. However, any further increase in the dose tended to reduce the release rate slightly and to prolong the plateau level; the reason for this is not clear. Serum levels and urinary excretion of the peptide in rats after repeated s.c. injection of the msp every 4 weeks exhibited similar profiles after each injection; no changes in absorption and excretion could be demonstrated after repeated injection [29].

In clinical studies, the msp formulation containing PLGA(75/25)-14,000 and about 8% leuprorelin was demonstrated to provide linear sustained release and, as expected, successfully achieved persistent serum drug levels over 4 weeks after i.m. injection. These levels correlated well with those obtained in the animal studies [39].

4.2. Three-month depot formulation

About 50 kinds of leuprorelin msp were prepared using PLGA(75/25) with a M_w of 9100–23 000, PLGA(90/10) with a M_w of 9200–22 900 and PLA with a M_w of 4700–162 100. Their drug release profiles were assessed in in vitro and in vivo tests

[36]. For these long-term depot msp formulations, the in vitro release profile, which included a long initial lag time followed by relatively rapid release, were considerably different from the in vivo ones. Most of the msp released the drug relatively linearly in vivo, mainly in parallel with polymer erosion. The in vitro release test using 1/30 M phosphate buffer, pH 7.0, containing 0.1% Tween 80 at 37°C could not be used to assess the 3-month depot formulation.

Preliminary in vivo studies indicated that msp made using PLA-18,200, PLA-21,500 or PLGA(90/10)-19,000 provided slightly delayed but continuous drug release over 3 months without a lag time. Msp prepared with PLGA(90/10)-12,000 released the drug rather more rapidly over the initial 2 weeks, but those made with PLA-10,000 or PLA-18,000 provided sustained release for 13 weeks. Finally, we focused on PLA with a M_w of about 12 000–18 000 and determined the in vivo release of several kinds of msp made with these PLA in detail, including formulations produced on a large-scale. As shown in Fig. 9, all these msp provided linear sustained release and adequately persistent serum drug levels (about 2 ng/ml at a dose of 100 μg/kg/day) for over 13 weeks. The release rates (the slope of each remaining drug profile) of these msp were almost the same, but initial release was slightly enhanced by a decrease in the molecular weight. Thus, msp made with PLA-15,000 (the average of these M_w), containing less than 0.1% water-soluble oligomers and loaded with 12% drug were considered to be the most desirable for the 3-month depot formulation. In a formulation study using 20 kinds of PLA with molecular weights of 8700–54 700, the water-soluble oligomer content of PLA was found to strongly influence the initial burst of drug release from the msp: an increase in the amount of oligomers produced a proportional increase in this initial burst [36]. The first-day burst was reduced to less than 10% when PLA containing less than 0.1% oligomers was used. This water-soluble acid fraction was composed of heptamers or smaller oligomers of lactic acid and was assumed to interfere with the formation of the hydrophobic barrier by arranging the polymer around the drug domains as shown in the next section, and by increasing the number of aqueous channels through the polymer barrier, resulting in a 'tunnel effect'. Reduction of the amount of these oligomers in the polymer is therefore as essential for the 3-month depot preparation as for the 1-month depot formulation containing PLGA.

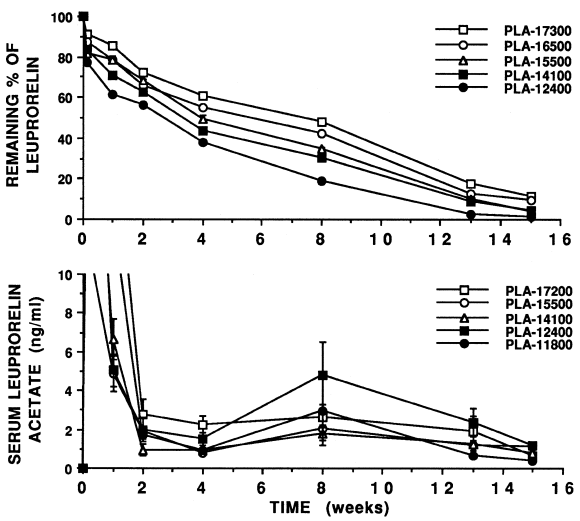


Fig. 9. Remaining percentage at the injection site and serum levels of leuprorelin acetate in rats after s.c. injection of 3-month depot PLA microspheres [36] (dose = 4.05 mg/rat, mean ± S.E., $n = 5$).

An in vivo release study of PLA msp charged with different amounts of drug (9–18%) showed that msp charged with 15 and 18% drug produced rapid initial release, probably attributable to an increase in the number of aqueous channels formed by the hydrophilic drug as observed with the 1-month depot msp. This indicates that drug loading should be limited to about 12%, and the load is set at about 11% in the final product. The particle size of the microspheres also had a definite effect on the release rate; smaller particles produced more rapid initial release. However, fine msp with a similar size distribution of about 20 μm and which produced reproducible continuous release for 3 months were obtained using a well-regulated manufacturing process similar to that used for the 1-month depot formulation.

Serum leuprorelin levels in rats and dogs were well sustained over 13 weeks following a short initial elevation after s.c. and i.m. injection of the 3-month depot msp with PLA-15,000 [36,37]. The serum plateau levels attained after injection of the 3-month depot msp preparation were slightly lower than those after injection of the same dose of the 1-month depot preparation. The reason for this remains unclear at present, but is probably ascribable to the slightly larger initial release over the first week and the larger

amount of drug remaining in the msp after 13 weeks (sustained release over more than 3 months), and possibly due to the more rapid clearance of PLA msp by blood scavenger cells in comparison with PLGA msp.

5. Mechanisms of drug entrapment and release

Continuous long-term control of the release of this water-soluble peptide and of the reproductive system for 1 or 3 months was achieved by incorporation of leuprorelin into biodegradable polynuclear reservoir-type mcp in which fine drug cores were dispersed within a polymer matrix.

The T_gs of PLA-14,100, PLGA(74/26)-13,700 and msp prepared using these polymers and charged with 0–8% drug were determined (Fig. 10) [36]. Formation of msp with or without leuprorelin caused a distinct elevation in T_g for both polymers, and the T_g of the msp increased gradually with increasing peptide load. With a low peptide charge, the trapping efficiency was somewhat low, maximum entrapment being observed at drug loads of between 5 and 8%, and first-day release was high. We hypothesized, as shown in Fig. 11, that a rigid structure was formed in the leuprorelin msp, that is, the polymer (PLGA or PLA) molecules were arranged around the drug cores in a similar way to surfactant molecules in a micelle, due to ionic interaction between the basic amino acids of the drug and the terminal carboxylic anions of the polymer. Thus, a barrier against diffusion of the hydrophilic drug was created by the hydrophobic

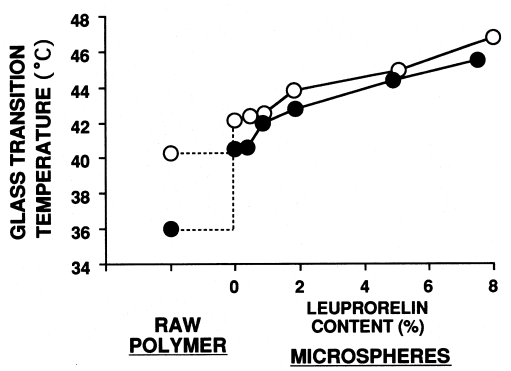


Fig. 10. Glass transition temperature of PLGA(74/26)-13,700 (●) and PLA-14,100 (○) microspheres containing various amounts of leuprorelin [36].

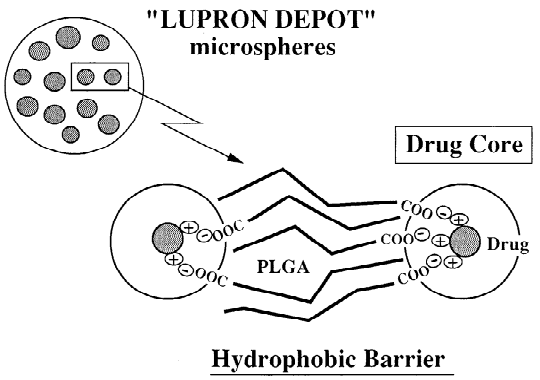


Fig. 11. Hypothetical schema of the hydrophobic diffusion barrier in leuprorelin PLGA and PLA microspheres [32].

long alkyl chains of the polymer. The T_g of msp containing a lipophilic drug such as cyclosporin A, which does not interact chemically with the polymer and is merely dispersed or dissolved in the polymer matrix at a molecular level, decreases with drug loading [118]. The elevation of T_g in msp formed without the peptide could therefore result from arrangement of the polymers at the interface between the msp and the outer water layer.

An ionic interaction between the polymers and leuprorelin was confirmed by a chemical shift in the arginyl and histidyl protons of the peptide to a lower magnetic field in the NMR spectra of the W_1/O emulsion (unpublished data). However, no diffraction peak was observed on powder X-ray diffraction analysis of these msp, even with a high drug content. The formation of such a rigid structure is also supported by the increase in viscosity of the W_1/O emulsion with increased drug loading. Addition of acids such as citric and tartaric acid, which are more acidic than PLA and PLGA but not acetic acid to the inner drug cores caused a decrease in the trapping efficiency and markedly increased initial burst release from the msp due to interference with the ionic interaction between the peptide and the polymer (unpublished data). These phenomena were observed more clearly in msp containing thyrotropin-releasing hormone (TRH, 5-oxo-Pro-His-Pro-NH_2) which is a small water-soluble weakly basic peptide [116]. Drug release from TRH msp was markedly influenced by the load and the salt of the drug employed. Msp made using PLGA(75/25)-14,000 and loaded with 1% TRH or TRH tartrate produced a fairly large

initial burst (first day release); indeed, a 100% burst was observed for the latter. However, msp containing TRH as the free base were found to attain better release control over a 3-week period than those containing the tartrate salt. The percentage retained on the first day in the in vitro release test increased with drug loads of up to 7.0% then decreased with further increases in the drug load. The trapping efficiency was, on the whole, correlated with the percentage retained on the first day. An increase in drug load generally leads to partial destruction of the W/O emulsion and an increase in the number of aqueous channels in the polymer matrix, resulting in a decrease in entrapment and an increase in the initial burst as described for the leuprorelin msp. The large initial burst from msp loaded with 1% TRH could not be explained by conventional studies because msp with low loads must have a thicker physical barrier against diffusion of the water-soluble peptide than highly loaded msp. A satisfactorily small burst and high trapping efficiency were attained at loading amounts ranging from 2.4–9.1%. Msp loaded with 7% TRH exhibited the smallest initial burst followed by constant release over 4 weeks.

Msp prepared with TRH tartrate released all of the incorporated drug after 1 day, irrespective of the drug load (1.0–9.0%) [119]. Addition of various acids to the inner water layer containing TRH as the free base also dramatically increased the initial burst. When especially strong acids such as hydrochloric, tartaric or citric acids were added, almost all of the drug was released after 1 day. These results indicate that the same amounts of TRH free base are necessary to gather the polymer around the drug core, rearrange the polymer alkyl chains, and construct rigid barrier walls against drug diffusion, based on the ionic interaction between the drug and PLGA as shown in Fig. 11. The influence of the drug load is much stronger than that for the leuprorelin msp because TRH is a smaller peptide which can diffuse more easily through the swollen hydrated polymer matrix. It is also a weaker base and thus exerts a weaker interaction with the polymer carboxylic acid residues. The viscosity of the W/O emulsion increased markedly on increasing the drug load from 1 to 9.1% and decreased on addition of tartaric acid. The increase in the initial burst and decrease in the trapping efficiency observed at lower or higher drug loads was accelerated by a decrease in the M_w of the polymer used as a result of thinning of the hydrophobic barriers against diffusion of the peptide [120].

Pradhan and Vasanada [121] reported the release rate of triglycine from PLA msp. In contrast to TRH, this hydrophilic peptide exerts no ionic interaction with the polymer. These msp provided rapid release (80% within 3 days), independent of polymer erosion, mostly by diffusion through the matrix and the release rate increased as the peptide load was raised (2.5 to 10%). Recently, in order to improve the trapping efficiency and decrease the initial release of neutral or acidic drugs such as methotrexate (MTX), we have successfully developed a novel encapsulation method in which a basic amino acid such as arginine or lysine is added to the inner drug cores [122,123]. The initial release of MTX from PLGA msp containing 3% lysine was reduced dramatically from 85.5 to 7.6%, and subsequent release was well controlled by erosion of the polymer, remaining constant for 1 month when PLGA(75/25)-14,000 was used. The viscosity of the W/O emulsion increased markedly during the process of in-water drying with the addition of basic amino acids, indicating the formation of a rigid structure surrounding drug cores containing basic amino acids. However, the loading limit of the drug was not large because the water-soluble basic amino acid had already been added to the inner drug core at a concentration of 2–5%. These results could also explain why such water-soluble peptides are efficiently entrapped during the in-water drying process and why drug release from msp is controlled mainly by erosion of the polymer over long periods, avoiding a large initial burst.

The composition of the release medium in the in vitro release test strongly influenced the drug release as described above. The release rate for leuprorelin msp was enhanced by decreasing the pH and elevating the temperature of the medium due to the promotion of polymer hydrolysis. A decrease in the osmolarity of the medium also caused an increase in peptide release even though no significant change in PLGA degradation was observed; this rapid release was probably attributable to enhanced exudation of the water-soluble drug due to increased water penetration from the outer medium and/or destruction of the polymer wall, resulting from the large osmotic difference between the inner drug core and the outer

release medium. In order to ensure quality control when manufacturing the 1-month and 3-month depot products, different in vitro short-term (<30 h) release tests were devised for each product. These involve enhancing the drug release rate by altering the conditions, for example, using a higher medium temperature or gradually elevating the medium temperature, acidifying the medium, or reducing its tonicity (unpublished data). These tests allow long-term effects to be detected within short periods.

6. Pharmacological effects in animals

6.1. In male animals

Prostate cancer is now the second leading cause of cancer death in men, exceeded only by lung cancer. It is estimated that approximately 317 000 new cases and 40 400 prostate cancer-related deaths occurred in the US in 1996. This cancer accounts for 36% of all male cancers and 13% of male cancer-related deaths. Incidence rates have increased significantly over the past 35 years, most likely as a result of increased early tumor detection due to the increased availability of prostatic-specific antigen (PSA) screening. The number of patients diagnosed at Stage D is decreasing at 2% per year also due to earlier diagnosis. The incidence rate in Japan is almost half that in the US. Over 80% of these cancers are endocrine-dependent. They tend to grow slowly and can be detected at an advanced stage following bone metastasis in more than 50% of patients. The treatment of advanced prostate cancer (Stage D) focuses on hormonal therapies to reduce androgen levels or block their effects, and includes surgical (orchiectomy) and medical (diethylstilbestrol, LH-RH agonists (30%) and antiandrogens) castration.

Suppression of gonadotropin and sex hormone serum levels and changes in weight in the genital organs were determined in rats and dogs after injection of the 1-month depot formulation of leuprorelin. Striking suppression of serum LH, FSH and testosterone were induced in rats or dogs for 6 weeks, following an initial elevation (flare-up) of the levels of these hormones, by a single injection [27,30,110]. The initial flare-up of serum testosterone disappeared completely after 3 days in rats [27,30] and after 2 weeks in dogs [110] and humans [39]. On repeated injection, the second and succeeding injections caused hardly any flare-up as long as chemical castration was adequately maintained by the previous injection (Fig. 8) [30]. As shown in Fig. 12, the msp produced marked regression of the

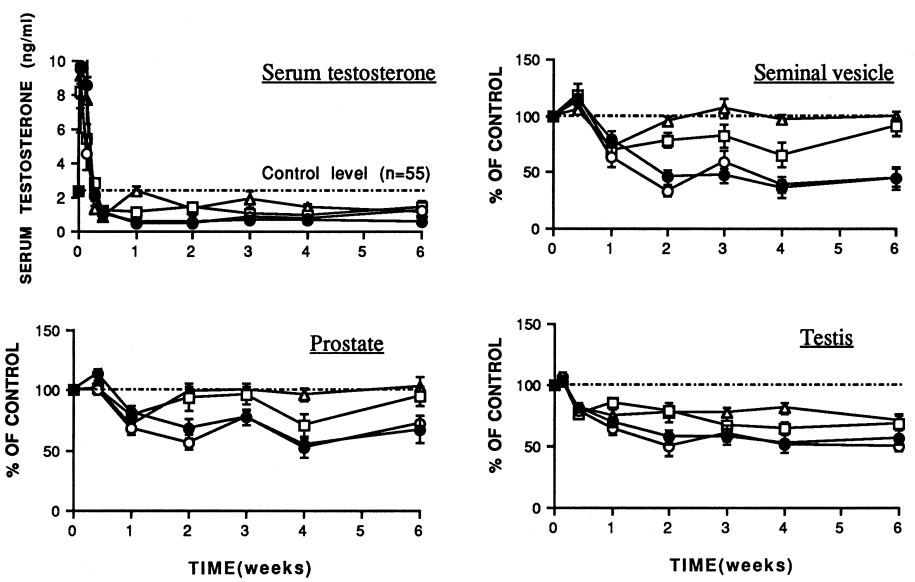


Fig. 12. Serum testosterone and weight change in genital organs of rats after i.m. (clear) and s.c. (solid) injection of leuprorelin 1-month depot microspheres [110] (dose = 1 (△), 10 (□), 100 (○, ●) μg/kg/day, mean±S.E., $n = 5$).

genital organs (testis, seminal vesicles, and prostate), a marker of prostate tumor suppression, in rats within 2 weeks. This effect lasted for over 6 weeks after a single injection of more than 100 μg/kg/day [27,110]. The adrenal gland, which also produces testosterone but is not regulated by LH-RH, was not affected by the msp treatment [27]. A periodic challenge test using a solution of the peptide (100 μg/kg) also revealed that a single injection of the msp caused dramatic suppression of the ability of the pituitary-gonadal system to secrete gonadotropin and testosterone for over 5 weeks (Fig. 13) [30]. Complete recovery of these functions was observed 10 weeks after the injection; thus, chemical castration was proven to be only temporary.

In these preclinical animal studies, leuprorelin msp containing PLGA(75/25)-14,000 were shown to maintain persistent serum drug levels for over 4 weeks after injection and achieved reliable pharmacological effects. This led to the expectation of adequate medical efficacy. We estimated the optimal dose for clinical studies from these results in animals [29]. Serum levels of 0.4 to 1 ng/ml produced sufficient suppression of serum testosterone and genital organ weight in rats after constant infusion using an osmotic minipump [27]. Assuming no species differences in responsiveness, the effective human dose was calculated to be 3.2–8.1 mg per month ($8.1=1\times10^{-6}$ (ng/ml)$\times2.89\times10^{3}$ (ml/day/kg)$\times30$ (day)$\times70$ (kg)/0.75 (%)) based on the Cl_{tot} for humans (2.89 l/day/kg), body weight (70 kg) and the BA of the msp over 4 weeks (75%, calculated using the initial burst and the amount remaining 4 weeks after injection). The dose of the 1-month depot is 7.5 mg in the US and 3.75 mg in the EU and Japan for patients with advanced prostate cancer; these doses coincide with the calculated doses. The first clinical study, carried out in patients with advanced (Stage D_2) prostate cancer in the US, demonstrated that plateau serum drug levels persisted for over 4 weeks after a single injection of the msp and that dramatic suppression of serum testosterone to below castration levels occurred with 4-weekly repeated injections at a dose of 7.5 mg, corresponding to 1/4 of the dose needed for a conventional injection of peptide solution [39].

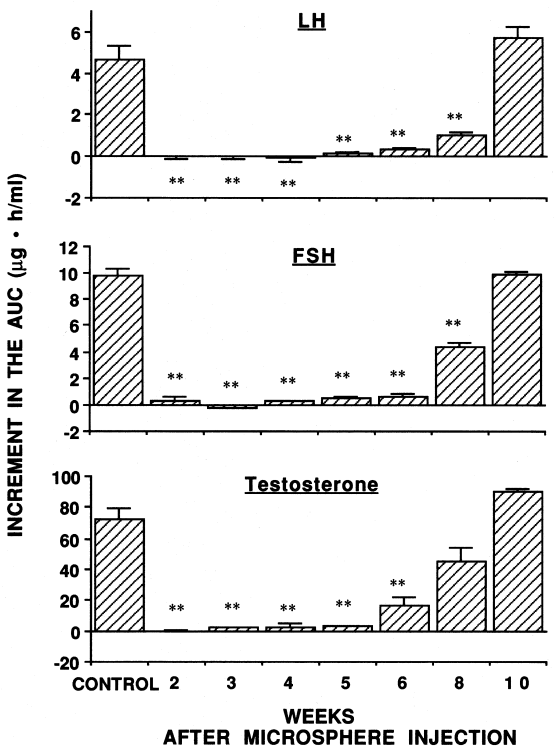


Fig. 13. Serum LH levels and increase in the AUC of serum LH, FSH and testosterone for 8 h in rats following challenge with leuprorelin solution at different times (challenge test) after s.c. injection of 1-month depot microspheres [30] (dose=100 μg/kg (solution) and 1.35 mg/rat (depot), mean±S.E., $n=5$, $^{**}P<0.01$).

Serum testosterone levels in rats and dogs were severely suppressed for 17–18 weeks after a single s.c. or i.m. injection of the 3-month depot msp at a dose of 100 (rat) or 25.6 (dog) μg/kg/day, as with the 1-month depot. Normal testosterone levels were again observed in both species 20–22 weeks after injection. The msp also caused drastic suppression of serum LH and FSH levels for 21 weeks, following an initial transient elevation due to the agonistic activity of the drug, in rats after s.c. or i.m. injection at a dose of 100 μg/kg/day. Persistent 'chemical castration' for 3 months was confirmed to be produced by the msp by periodic challenge tests with the peptide solution and determination of genital organ growth after injection of the msp. As shown in Fig. 14, the release of LH, FSH, and testosterone was depressed for 15 weeks by a single injection of the msp, to the same extent as by three repeated treatments with the 1-month depot. Pituitary-gonadal function partially recovered by 18 and 21 weeks after injection, but was still significantly suppressed.

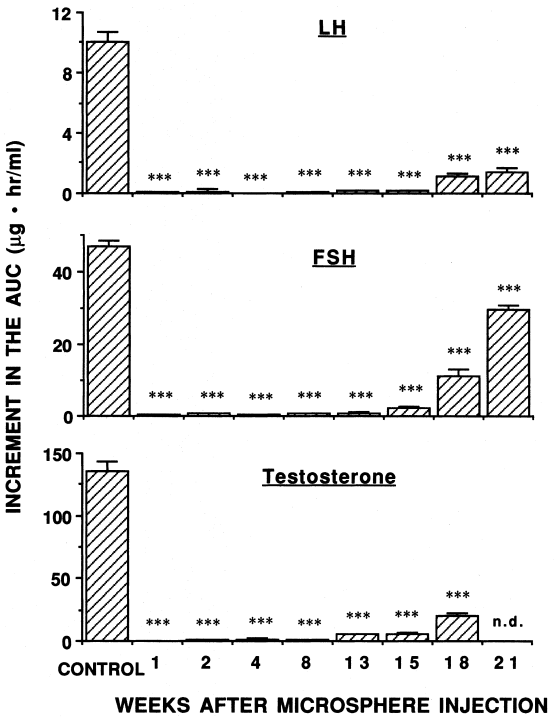


Fig. 14. Increase in the AUC of serum LH, FSH and testosterone in rats following a challenge test after i.m. injection of leuprorelin 3-month depot microspheres [37] (dose = 100 μg/kg (solution) and 4.5 mg/rat (depot), mean ± S.E., $n = 5$, ***$P < 0.001$, n.d.: not determined).

Growth of the testis was inhibited dose-dependently at doses of 1–100 μg/kg/day and was strongly suppressed even by the lowest dose. This suppression profile correlated fairly well with that for serum testosterone (Fig. 15). Growth of both the seminal vesicles and the prostate was also depressed dose-dependently, after a slight initial increase. The lowest dose was insufficient to cause suppression, but 10 and 30 μg/kg/day produced obvious inhibition for over 13 weeks. In all of these reproductive organs, the strongest inhibition was achieved at a dose of 100 μg/kg/day, as with the 1-month depot formulation.

6.2. In female animals

Endometriosis is a disease caused by the growth of aberrant or ectopic endometrium at various locations within the pelvic cavity, including the ovaries, uterine ligaments, rectovaginal septum and pelvic peritoneum. The disease occurs in ~10% of all women of reproductive age and is a common cause of chronic pelvic pain and/or infertility. Prior to the development of LH-RH agonists, the therapeutic options included conservative surgery or medical suppression with the androgen danazol. In the US, the safety and efficacy of leuprorelin depot (3.75 mg) in patients with endometriosis was first assessed using six injections every 4 weeks [54]. Dysmenorrhea, pelvic pain and pelvic tenderness all responded significantly to treatment with the msp. Estradiol levels decreased significantly to menopausal levels (<30 pg/ml) and the menses were completely suppressed.

We determined the effect of medication with the 1-month depot msp on endometriosis using female rats and Jones' experimental rat model [124]. Sustained serum levels of the peptide and persistent suppression of LH and FSH were obtained over 4 weeks after a single injection of the msp at doses of more than 100 μg/kg/day. Serum estradiol levels were also suppressed for 6 weeks after injection without any initial flare-up, and the maximal inhibitory levels after 3 weeks were close to those in ovariectomized female rats. The experimental procedure used for the surgical induction of endometriosis in rats and typical responses after s.c. injection of the 1-month depot msp are shown in Fig. 16 and Fig. 17, respectively [26]. A single treatment with the msp at a dose of 100 μg/kg/day produced striking regression of the growth of endometrial explants (92% regression and 54% disappearance) 3 weeks after injection (Fig. 17), comparable with that achieved by surgical ovariectomy. At a dose of 10 μg/kg/day, only two of five rats showed signs of regression. Daily intermittent s.c. injection of the peptide solution (100 μg/kg) significantly suppressed the growth of all explants, although they still remained visible. Daily nasal administration of the peptide (100 μg/kg) with 5% α-cyclodextrin (an absorption promoter) exerted a significant inhibitory effect, which was almost identical to that of the msp at a dose of 10 μg/kg/day. Daily cytological examination of vaginal smears following injection of the msp also revealed reversible and sustained suppression; the estrous cycle was arrested in diestrus for 6 weeks after treatment and recovered 8–10 weeks later.

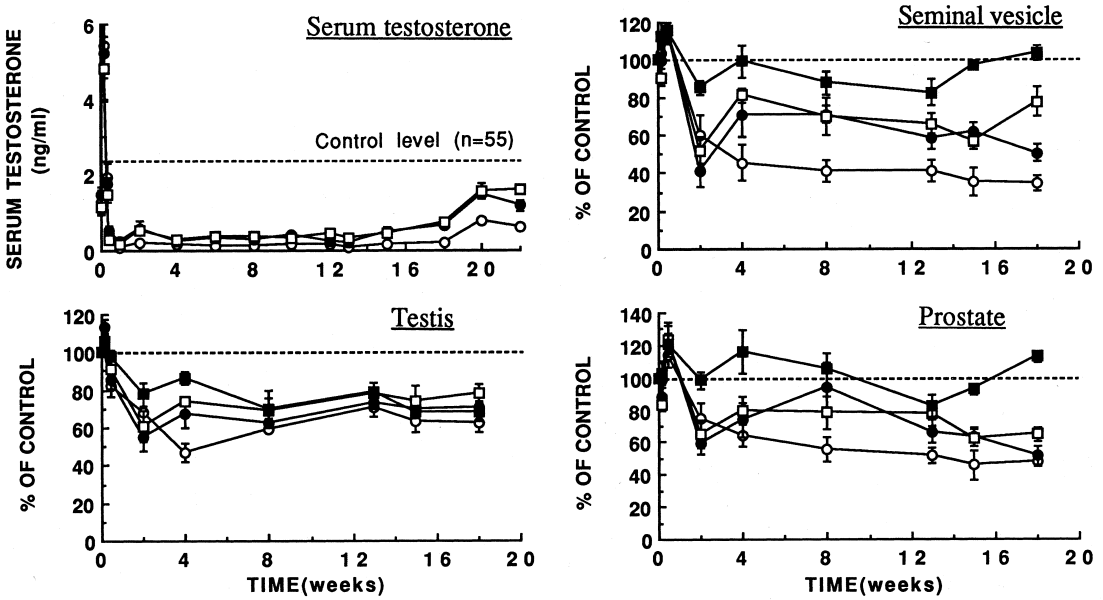


Fig. 15. Serum testosterone and weight change in genital organs of rats after i.m. injection of leuprorelin 3-month depot microspheres [37] (dose = 1 (■), 10 (□), 30 (●), 100 (○) μg/kg/day, mean ± S.E., $n = 5$).

Suppression of serum LH, FSH and estradiol levels and genital organ weight were also evaluated in female rats after injection of the 3-month depot formulation [38]. In female rats confirmed to have synchronized estrous cycles by vaginal smear checks, sustained serum drug levels and definite suppression of serum LH, FSH and estradiol were confirmed to be produced for over 16 weeks by a single injection of the msp at a dose of 100 μg/kg/day. The same degree of suppression of LH and FSH levels was attained with doses of 1, 10 and 100 μg/kg/day, but the suppression of estradiol was insufficient at a dose of 1 μg/kg/day. As shown in Fig. 18, an injection of the msp suppressed growth of the ovaries and the uterus in a dose-dependent manner; a dose of 1 μg/kg/day was insufficient to suppress uterine growth, while 100 μg/kg/day produced the strongest growth suppression in both organs. These responses correlated well with serum estradiol levels. Serum LH and FSH responses in a challenge test indicated that complete suppression was sustained for 16 weeks (about 4 months) (Fig. 19), as in male rats. While evaluating the serum estradiol response, we were unable to demonstrate any dysfunction of ovarian steroidogenesis due to lack of elevation after challenge in normal untreated rats or rats treated with the msp.

These results suggest that the 3-month depot formulation may be more convenient than the 1-month depot, and since it would ensure greater patient compliance, it would result in better therapeutic effects in patients suffering from hormone-dependent diseases. Clinical studies carried out in patients with advanced prostatic cancer in the US and EU have confirmed good objective results [50,51]. A 6-month depot formulation has now been investigated and the possibility of commercial preparation is under consideration (patent applied for).

Therapeutic peptides and proteins, such as growth factors, regulatory factors, hormones and cytokines, are regulated by complex feedback systems with numerous controlling influences that maintain homeostasis in the body. Their mechanisms of action, pharmacokinetics, pharmacodynamics and receptor dynamics must be defined to establish rational dosage regimens and optimize the design of the delivery system, so as to maximize their therapeutic effects and minimize adverse reactions. For example, the induction of ovulation by LH-RH is known to require pulsatile infusions at 90-min intervals (1

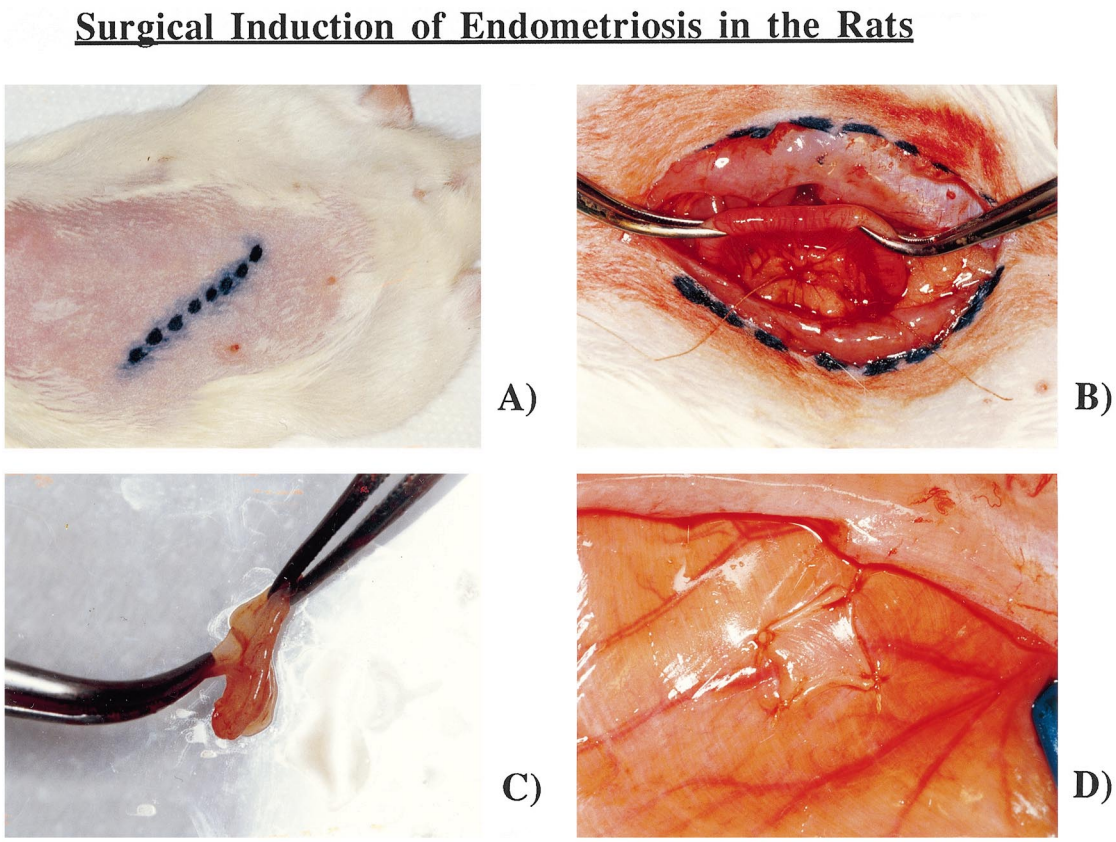


Fig. 16. Procedure for induction of experimental endometriosis in female rats by Jones' method [26,124]. (A) 5 cm oblique incision of the body wall, (B) ligation of uterine blood vessels and uterine segment excised, (C) myometrium separated from endometrium, (D) 5×5 mm endometrial segment sutured to body wall of peritoneal cavity.

pulse per h) to achieve maximum agonistic activity [125], whereas the suppression of hormone-dependent diseases by LH-RH analogs needs constant release to allow persistent receptor downregulation, as described in our studies. In studies using human calcitonin (hCT) in rats, we found that plasma calcium levels were depressed in response to each injection when a pulsatile sequence of s.c. injections, for example, twice a week or three times a day, was administered but that there was only an initial single response when hCT was given by constant infusion over 10 days. Continuous infusion of hCT with an osmotic minipump over 3 weeks did not inhibit bone resorption (reduction of femoral bone density) in ovariectomized female rats but paradoxically promoted it (unpublished data). This experiment indicated a very important consideration in the design of controlled release mechanisms using biodegradable msp. As a consequence, we recommend pulsatile release but not constant prolonged release of CT and parathyroid hormone (PTH) for the treatment of hypercalcemia and osteoporosis. However, a continuous s.c. infusion of the somatostatin analog (SMS201-995) produced more complete lowering of growth hormone (GH) plasma levels in acromegalic patients than three daily s.c. injections yielding the same total daily dose [126]. Constant infusion of hGH exerted a dose-dependent pharmacological effect comparable to that observed after daily bolus injection [127]. Recently, the enhancement of its pharmacological activities by a single injection of the msp containing rhGH as comparison with the daily

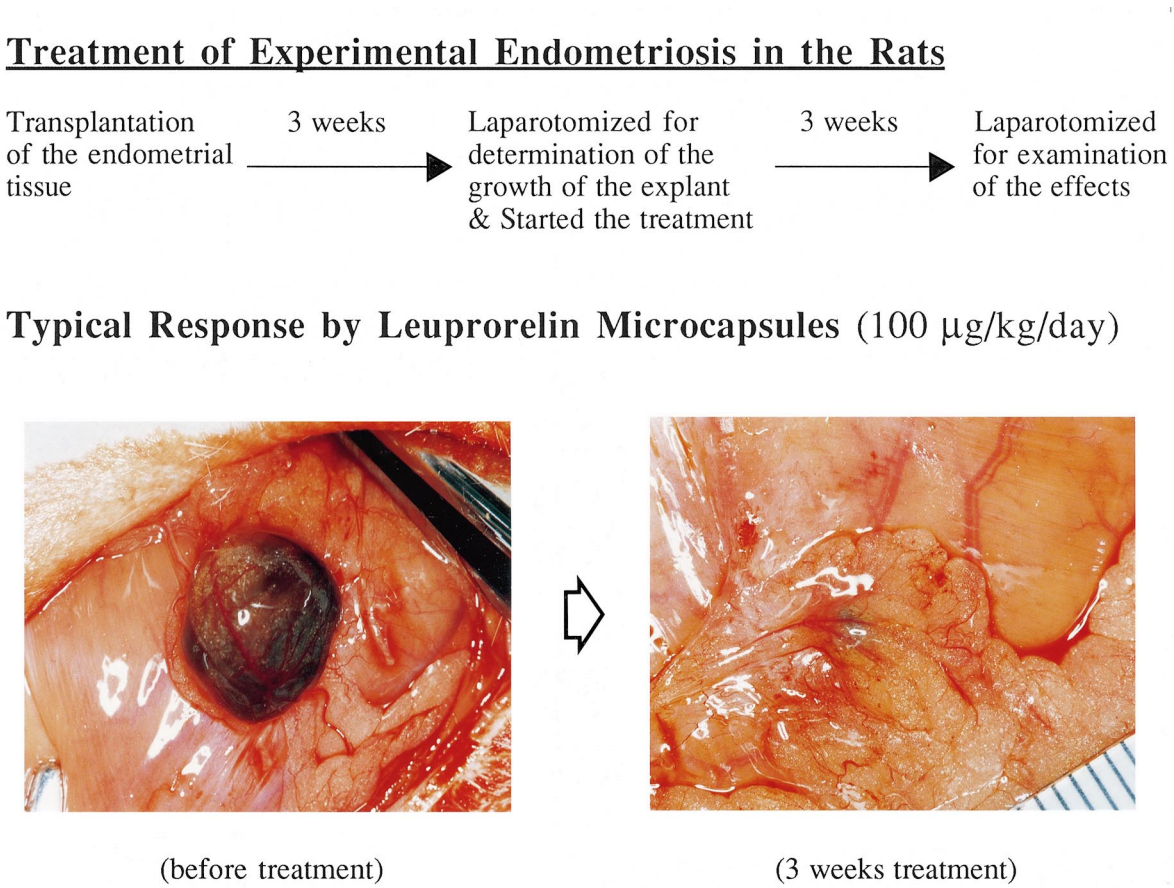


Fig. 17. Treatment procedure for experimental endometriosis in female rats and its typical response after 3 weeks of treatment with leuprorelin 1-month depot microspheres [26] (dose = 0.81 mg/rat). Endometrial explant before treatment shows growth of a well vascularized endometrial segment with fluid and a typical chocolate cyst, whereas after 3 weeks of treatment, the explant has regressed and has a sheath of connective tissue but no fluid.

injection of the same amount of rhGH was proved in monkeys [106]. Furthermore, erythropoietin, a regulator of the production of red blood cells, also produced a significantly better biological response when administered by continuous transendometrial application using an osmotic minipump than following pulsatile administration [128]. In addition to the consideration on these receptor dynamics, most endogenous peptides and proteins have a circadian rhythm and their activities change depending on the time of administration (chronotherapy). Thus, pulsatile release and triggered release controlled systems rather than constant release devices are required for these highly regulated peptides and proteins.

7. Conclusions

Many biodegradable polymers obtained from natural sources or by chemical synthesis are useful as drug carriers for drug delivery systems which rely on three mechanisms: mucosal absorption, controlled release, and targeting [33]. Of these polymers, PLA and PLGA were concluded to be the most desirable for controlled release delivery systems because of their excellent biocompatibility, the easy and precise control of their erosion rates by regulation of their molecular weights and the LA/GA copolymer ratio, the lack of necessity for crosslinking agents during microencapsulation, and other physicochemical and

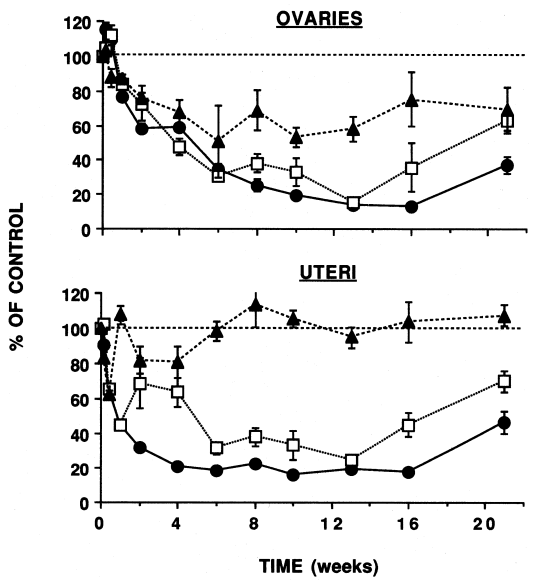


Fig. 18. Weight change in genital organs of female rats after s.c. injection of leuprorelin 3-month depot microspheres [38] (dose = 0.036 (▲), 0.36 (□), 3.6 (●) mg/rat, mean±S.E., $n=5$).

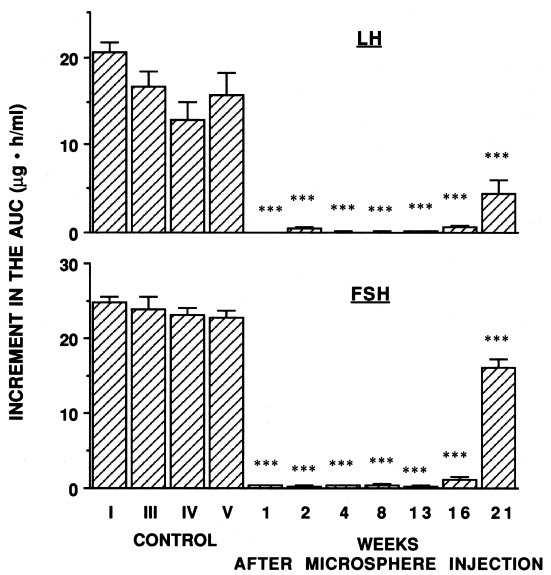


Fig. 19. Increase in the AUC of serum LH and FSH in female rats following a challenge test after s.c. injection of leuprorelin 3-month depot microspheres [37] (dose = 100 μg/kg (solution) and 3.6 mg/rat (depot), mean±S.E., $n=5$, ***$P<0.001$, untreated control: I, proestrus; III, estrus; IV, metestrus; V, diestrus).

biological characteristics as described above. Moreover, the highly effective combination of an anionic polymer and a cationic drug (such as leuprorelin or TRH) has allowed linear sustained release to be achieved for long periods of 1–3 months without dose dumping. This release is well controlled by polymer erosion from the simple msp matrix system, probably due to the formation of a rigid structure with polymer alkyl chains surrounding the water-soluble drug cores. Thus, we have been able to create epoch-making new drug forms which persistently exert their pharmacological activity over long periods after a single injection. As a result of our aggressive and persistent pursuit of mucosal delivery systems for therapeutic peptides since 1976 and of controlled release dosage forms since 1980, the pharmacological effects of this superagonist on the pituitary-gonadal system were found to be enhanced by continuous stimulation after vaginal administration or infusion. This hinted that a sustained release injection would be a rational dosage form and led us to devise the novel microencapsulation method of in-water-drying through use of a W/O/W emulsion for water-soluble peptides, resulting in the development of a commercial product. This ideal leuprorelin delivery system, the 1-month depot injection Lupron Depot®, was first marketed in the US in 1989. The 3-month depot was approved in the US in 1995. These depot formulations have undoubtedly improved patient quality of life (QOL) and compliance (90% in prostate cancer), and have dramatically enhanced the medical usefulness of LH-RH superagonist leuprorelin acetate. Furthermore, this success highlights the roles of biodegradable polymers, microsphere formulation and novel drug delivery systems in attaining highly efficient new drugs.

However, in order to achieve a more rational medical approach for most therapeutic peptides and proteins, drug delivery systems providing more complex release control (such as stimulus mediated or sensor control), which would take into account circadian rhythms or disease symptoms by using ‘intelligent’ materials responsive to external stimuli, are necessary. More complex msp resembling viruses or living cells which would act as self-producing drug systems without the need for periodic drug supply would be ideal. Therefore, our success with long-term, zero-order release depot formulations of peptides might be regarded as a first step in the

development of controlled delivery systems for medical use involving biodegradable msp.

Acknowledgements

The author greatly thanks Mr. Masaki Yamamoto, Dr. Toshiro Heya, Ms. Yayoi Doken, Dr. Yasuaki Ogawa and Dr. Takatsuka Yashiki for excellent collaboration, Drs. Hajime Toguchi, Tsugio Shimamoto, Hiroyuki Mima, Masahiko Fujino, Yukio Sugino and Sadao Iguchi for persistent encouragement, and Dr. David B. Douglas for valuable comments about the manuscript.

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