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Journal of Controlled Release 89 (2003) 297–310
journal of controlled release
www.elsevier.com/locate/jconrel

Lipid microparticles as sustained release system for a GnRH antagonist (Antide)

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Received 15 November 2002; accepted 11 February 2003

Abstract

Lipid microparticles (LMs) as a sustained release system for a gonadotropin release hormone (GnRH) antagonist (Antide) were prepared and evaluated. Antide loaded microparticles (Antide–LMs) were obtained by a cryogenic micronization process starting from two different monoglycerides (glyceryl monobehenate and glyceryl monostearate) and using two different incorporation methods (co-melting and solvent evaporation). Antide–LMs, 2% (w/w) loading, were characterized for drug incorporation by RP-HPLC, particle size by laser diffractometry and surface morphology by scanning electron microscopy. In vitro peptide release and in vitro biological activity were also studied. Serum Antide and testosterone levels, as pharmacodynamic marker, were assessed following subcutaneous administration in rats. Antide–LMs showed a mean diameter of approximately 30 μm and variable Antide release depending on lipid matrix and incorporation method. In vivo experiments demonstrated that detectable Antide plasma levels were present, in the case of Antide–LMs based on Compritol E ATO obtained by co-melting procedure, for at least 30 days after dosing. Testosterone levels were consistent with prolonged pharmacokinetic profiles. In vitro release of Antide from LMs correlated well with the in vivo release. In conclusion, LMs can sustain the release of Antide for at least 1 month. The levels of the initial 'burst' and the extent of the pharmacodynamic effect can be influenced by the lipid characteristics and by process conditions.

Keywords: Lipids; Microparticles; Sustained delivery; Antide

1. Introduction

Microparticles based on biodegradable polymers have been extensively studied for improving the efficacy of protein and peptide drugs and ameliorate patient compliance by reducing number of injections. Poly(D,L-lactide) (PLA) and poly(D,Llactide–co-glycolide) (PLGA) are the most characterized polymers for the preparation of biodegradable microparticles [1–4]. Microspheres of PLGA containing a GnRH agonist, Leuprolide (Leupron depot®) are available on the market for many years for the long-term treatment of prostatic cancer and endometriosis [5–7].

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0168-3659/03/$ – see front matter
doi:10.1016/S0168-3659(03)00120-2

On the other hand, physiological lipids (i.e. triglycerides, cholesterol, etc.), that are present in different body areas, have attracted increasing attention as alternative materials to polymers, showing potential advantages especially in terms of low toxicity and good biocompatibility [8]. However, few examples have been reported on the use of lipid microparticles (LMs) for peptides and proteins [9]. The use of lipid microparticles has been described in the veterinary field for sustained release of growth hormone in order to increase weight gain and milk production in treated animals [10]. More recently, lipid microparticles based on glyceryl tripalmitate, with particle size distribution suitable for subcutaneous and intramuscular injection (<150 μm), have been reported as a successful system for the in vitro sustained release of somatostatin, insulin and tymocartin [8,11]. The processes described in these studies, involving the use of chlorinated solvents, are very similar to solvent evaporation and precipitation methods also applied for the production of polymeric microparticles. However, even when the use of chlorinated solvents is not envisaged, the process could lead to some problems with respect to large scale production, considering the large volumes of dispersing media typically used in solvent evaporation and dispersion techniques.

Antagonists of the hypothalamic hormone, gonadotropin hormone-releasing hormone (GnRH), act by competitive blockade of the receptors producing an immediate and dose-related inhibition of gonadotropin release. Therefore, they can avoid the drawback of the GnRH agonists in inducing an initial stimulatory effect in hormone secretion and the relatively long period (2–3 weeks) of chronic exposure before complete suppression can occur [12]. GnRH antagonists have been studied for the treatment of various disorders which result from inappropriate sex hormone levels, including hormone dependent prostate and breast cancer, endometriosis, benign prostate hyperplasia and uterine fibroids. GnRH antagonists have been also employed in in vitro fertilization and embryo transfer (IVF–ET) programs to prevent a premature rise in LH [13,14]. However, for most of these indications the development of sustained release depot systems, for a variable time period, is required.

Antide, due to its in vivo potency and little histamine-releasing potential has become very promising for the treatment of fertility disorders and was subjected to clinical investigations [15–17].

The aim of our study was to investigate the potential of lipid microparticles as sustained release delivery system for Antide in all therapeutic indications where a gonadotropin suppression is required. Antide–LMs were prepared by an industrially scaleable process, i.e. cryogenic micronization, characterized in vitro and tested in vivo for pharmacokinetic (PK) and pharmacodynamic (PD) profiles. Special attention was addressed to the possibility of modulating in vitro/in vivo release kinetics by varying lipid matrix, physicochemical characteristics and preparation method. The capability of in vitro release kinetics to predict in vivo pharmacokinetic profiles was also evaluated.

2. Materials and methods

2.1. Materials

Antide, [*N*-Ac–D-Nal^{1}–D-Cpa^{2}–D-Pal^{3}–Ser^{4}–Lys(Nic)^{5}–D-Lys(Nic)^{6}–Leu^{7}–Ilys^{8}–Pro^{9}–D-Ala^{10}–NH_2], acetate salt was purchased from Bachem (Bubendorf, Switzerland). Glyceryl monobehenate (Compritol E ATO, with monoglyceride content >70%) was received as free samples from Gattefossè (Saint Priest, Cedex, France). Glyceryl monostearate (Imwitor 900 with a monoglyceride content of about 40%) was received from Condea (Witten, Germany). All the other reagents were obtained commercially as analytical grade samples.

2.2. Preparation of antide loaded lipid microparticles

Four Antide–LM batches were prepared according to proprietary technology, cryogenic micronization [18]. Antide was incorporated in monoglyceride matrices, Compritol E ATO and Imwitor 900, by using two experimental procedures—co-melting and solvent stripping. In the co-melting procedure, the drug was added under magnetic stirring to the molten lipid, kept in a thermostated bath at 85 °C in the case of Compritol E ATO and 75 °C in the case of Imwitor 900. Antide was allowed to dissolve for

approximately 2 h, until a clear solution appeared. In the solvent stripping method, the drug and the lipid were dissolved under stirring in a benzyl alcohol–ethanol mixture, 1:5 in the case of Compritol E ATO and 1:2 in the case of Imwitor 900, at 85 °C and 55 °C, respectively. The solution was then poured in Petri dishes and the solvent was evaporated under vacuum overnight. For all the preparations the Antide loading in the lipid matrices was 2% (w/w). The obtained lipid matrices, stored at −80°C, were micronized in a customized milling apparatus (MPX 348, Apex, Kent, UK) fluxing liquid nitrogen during the process. The obtained powders were sieved through a 125-μm sieve in an automatic sieving apparatus (AS 200 autosieving system, Retsch, Haan, Germany) and stored at 4 °C.

2.3. Particle characterization

2.3.1. Particle size distribution

Antide–LM particle size was assessed by laser diffractometry using a Malvern Microplus laser sizer (Microplus MAF 5001, Malvern, UK). The particles were dispersed in an aqueous solution containing 0.05% of Tween 20. Laser beam obscuration was at least 15%. The particle size results were expressed in micrometers as $D(v, 0.9)$, $D(v, 0.5)$, $D(v, 0.1)$ (meaning that 90, 50 and 10% of the particle sample volume is below the corresponding D value) and Span, or measurement of the width of particle size distribution. Span was calculated as follows:

$$\text{Span} = \frac{D(v, 0.9) - D(v, 0.1)}{D(v, 0.5)}$$

Experiments were performed in triplicate. The instrument was regularly checked with standard glass beads of 45±2 μm received from Alfatest (Rome, Italy).

2.3.2. Lipid microparticles morphology by scanning electron microscopy

The morphology of the particles was assessed before and after release test by scanning electron microscopy (SEM) on a DSM 940 scanning electron microscope (Zeiss, Germany). In particular, Antide–LMs prepared by the co-melting and solvent stripping procedures were incubated in phosphate buffer saline, pH 7.4, at 37 °C under stirring at 100 rpm. Residues were collected after 24 h, dried overnight in a desiccator containing silica gel, and stored at 4 °C. For SEM analysis, microspheres were attached to the specimen holder using double coated adhesive tape, and subsequently gold-sputtered for 3 min at 40 mA, in order to obtain a layer of approximately 30 nm of gold using an Emitech K550 coating unit.

2.3.3. Peptide content in the lipid microparticles

A 50-mg amount of Antide–LMs were first dissolved in 5 ml of acetone, shaken and sonicated for 2 min. A 5-ml volume of deionized water was then added to the acetone solution to extract the peptide. The obtained mixture was sonicated for 2 min and subsequently centrifuged for 15 min at 8000 rpm. The recovered clear solution was injected into HPLC equipment: 2690 separation module, 2487 dual absorbance detector (Waters, Milford, MA, USA). The peptide content in the microparticles was assayed with a gradient elution RP-HPLC method: C_{18} column Jupiter, 250×4.6 mm, 5 μm (Phenomenex, Hundsfield, UK); mobile phase A: 0.1 M phosphate buffer, pH 4.5; mobile phase B: acetonitrile; the linear gradient was increased from a 23% (v/v) phase B to 48% within first 30 min and decreased to the start conditions within 10 min. The flow-rate was 1 ml/min and the analysis was performed at room temperature. The UV detection was performed at 227 nm. Antide solutions of known concentrations (1–400 μg/ml) were used to generate standard curves.

All the experiments for peptide content were performed in triplicate.

2.3.4. In vitro release study

Antide release from microparticles was assessed in vitro by a dissolution method, using USP apparatus II. Antide–LMs (approximately 50 mg, accurately weighed) were transferred into a vessel containing 900 ml of deionized water stabilized at 37 °C under constant stirring at 100 rpm. At standardized time intervals (1, 2, 4, 6 and 24 h), 1-ml volume samples were withdrawn, filtered through 0.22-μm polysulfone filters (Acrodisc, Pall Gellmann Labs., Ann Arbor, MI, USA) and assayed for Antide content by RP-HPLC as described above. The filtering membranes were previously tested in order to exclude any interference with the peptide solution. An equal

volume of fresh medium was added after each withdrawal to reconstitute the original volume. The experiments were performed in triplicate.

2.3.5. In vitro bioactivity

The bioactivity of Antide after incorporation into LMs was determined with an in vitro bioassay. This method is based on the quantification of LH released from rat pituitary cells after incubation with a fixed quantity of GnRH, in combination with different concentrations of Antide [19]. Primary cell cultures were established starting from enzymatic digestion of pituitary glands removed from adult female rats. Recovered cells were plated at 2.5×10^5/well in 24-well plates (Falcon, Becton-Dickinson, Franklin Lakes, NJ, USA) and cultured for 72 h at 37 °C and 5% CO_2. Wells were washed three times and then treated for 24 h with in house reference Antide spiked in culture medium containing the relevant LM placebo (0.75, 3 and 12 ng/ml) or with Antide extracted from three Antide–LM formulations (1.5, 3 and 6 ng/ml). In each experimental session, samples and Antide reference material were tested in triplicate. Wells for basal and maximum level of secreted LH received culture medium alone. After 24 h incubation, monolayers were washed, samples and reference Antide dilutions were renewed and GnRH (10^{-8} M) was added to all the wells except to the basal wells that received an equal volume of culture medium. Conditioned medium from each well was collected after 4 h of incubation (37 °C, 5% CO_2) and stored at −20 °C until assayed for rat LH content by ELISA (Amersham Pharmacia Biotech). Results were expressed as percentage inhibition of LH secretion. The potency of each dilution was calculated by interpolating the LH inhibition value on the Antide reference linear/log regression curve. Then the percent of the recovery was determined dividing the estimated potency by the expected value and multiplying 100.

2.3.6. In vivo study

PK and PD of Antide–LMs were assessed in adult Sprague–Dawley male rats (~300 g). The microparticles were injected subcutaneously at a dose of 2 mg/kg, after reconstitution in a solution containing 5.6% (w/v) of glucose and 0.05% (w/v) Tween 20 using polypropylene syringes equipped with a 21-gauge needle. Microparticle concentration in the vehicle was about 45 mg/ml and the volume of administration was approximately 1 ml. As control, Antide bulk was used, dissolved in the same vehicle and at same dose level. A representative placebo LM, composed by Imwitor 900 matrix was also injected as a control for baseline testosterone levels. Three animals were used for each predefined time point. In the case of Antide–LMs, blood samples were collected at 0.5, 1, 2, 4, 8 h and at different terminal time points up to 29 days; for placebo LM terminal blood samples were collected on days 1, 4, 7 and 14; for the Antide control solution blood samples were withdrawn up to 96 h. Samples were collected in heparinized test tubes and plasma was separated by centrifugation (2500 *g*) at 4 °C and divided in two aliquots for PK and PD analyses. Antide levels were assessed using a HPLC–MS–MS method after solid-phase extraction of 0.5 ml of plasma on 96-well plate (Oasis HLB extraction cartridge, Waters, UK). Briefly, the HPLC–MS method (HPLC pump system TSP Spectrosystem P4000, autosampler TSP model AS3500 and mass detector LCQ Finnigan) was performed using column Symmetry 300 C_{18}, 5 μm, 2.1×150 mm (Waters). The mobile phase A was composed of 0.02% TFA in H_2O, while mobile phase B was 0.02% TFA in methanol. The gradient was increased from a 25% (v/v) phase B to 100% within first 10 min, kept constant for 3 min, and decreased to the starting conditions in 4 min. The flow-rate was 0.36 ml/min. The assay limit of quantitation was 1.4 ng/ml. Inter-assay precision was between 10 and 20%.

The pharmacodynamic marker testosterone was measured using radioimmunoassay (RIA) (Diagnostic Products, Los Angeles, CA, USA). The lower limit of detection for this assay was 0.2 ng/ml and the inter-assay precision was 12–15%. Pharmacokinetic parameters (area under the plasma versus time curve and terminal half life) were calculated by noncompartmental approach.

3. Results and discussion

The main goal of this study was to investigate whether lipids, especially monoglycerides could

incorporate a model peptide (Antide) and release it in vitro/in vivo with a sustained profile. At the same time, we aimed to develop a novel cryogenic manufacturing process to produce LMs [18]. Cryogenic micronization avoids the use of surfactants and of large quantities of dispersing media typical of solvent evaporation and dispersion techniques [8,11]. The cryogenic process can also reduce the exposure of labile drugs to high temperatures and organic solvents, lowering the risk of protein and peptide damage.

3.1. Lipid microparticles preparation

Antide was incorporated into lipid matrices by two processes: co-melting and solvent stripping. Antide–LM compositions, incorporation methods and relevant physicochemical data are reported in Table 1. Several lipids were assessed, the most promising being triglycerides with high content of monoglycerides, that are glyceryl monostearate (Imwitor 900®) and glyceryl monobehenate (Compritol E ATO®). Glyceryl monobehenate is a food grade approved excipient (E471). Although many examples have been reported for the corresponding triglyceride (Compritol 888 ATO®) [20,21], to our knowledge no specific applications for Compritol E ATO® have been reported in the pharmaceutical field. Nevertheless monostearate has been already reported as excellent solubilizer for a peptide like cyclosporin. In this case drug loadings up to 20–25% (w/w) were achieved applying co-melting procedure, reasonably due to the hydrophobic character of the compound [22]. With the same process, Antide, being more hydrophilic, was incorporated only up to 2% (w/w), both in glyceryl monobehenate and in glyceryl monostearate. Lipids enriched in triglycerides decreased the Antide loading capacity down to 0.01% (w/w). However, it was possible to increase Antide loading and expand lipid choice by introducing a solvent stripping procedure. In the latter method, the solubility of Antide and selected lipid in the organic mixture was the limiting step, while in the case of co-melting procedure the solubility of Antide in the lipid itself played a critical role. In addition, the solvent stripping method can avoid the use of high temperatures being particularly favorable for thermosensitive protein incorporation. In the case of Antide, due to its intrinsic resistance to thermal and mechanical stress, we could apply both techniques.

For the solvent stripping procedure, benzyl alcohol and ethanol were chosen. They are widely used in parenteral preparations [23], and more acceptable and less toxic than chlorinate solvents listed as class 2 solvents in the ICH guidelines [24]. Furthermore, benzyl alcohol dramatically increased the solubility of Antide being also a suitable solvent for the stripping method.

3.2. Particle size distribution and drug content

The size of microparticles, irrespective of the matrix material, has to be below 150 μm to be suitable for intramuscular or subcutaneous administration. All Antide–LMs prepared meet this requirement, showing a mean diameter of approximately 30 μm and an upper limit value $D(v, 0.9)$ of 120 μm (Table 1). However, distribution curves were relatively broad (Span was approximately 4.0 for all preparations) indicating that cryogenic micronization leads to a polydisperse system. Particle size was unaffected by LM compositions and by drug incorporation procedure. In order to eliminate larger particles that could cause needle blockage during the

Table 1
Antide–LM batch compositions and incorporation method, particle size distribution by laser difractometry [$D(v, 0.5)$; $D(v, 0.9)$; Span] and drug recovery (% w/w of drug recovered from the theoretical amount)

Batches	Compositions (% w/w) and incorporation methods	$D(v, 0.5)$ μm	$D(v, 0.9)$ μm	Span	Drug recovery (%)
LM1	Antide 2% Compritol E ATO stripped	29.0±2.0	98.8±11.5	3.3±0.2	87.2±1.2
LM2	Antide 2% Imwitor 900 stripped	29.1±2.9	111.1±15.5	3.7±0.1	89.0±1.0
LM3	Antide 2% Compritol E ATO comelted	24.4±1.2	115.3±4.9	4.6±0.1	92.5±0.8
LM4	Antide 2% Imwitor 900 comelted	34.6±5.2	112.8±21.6	3.1±0.2	94.5±0.8

in vivo administration, a sieving step was introduced (125 μm cut-off). The yield of the process considerably decreases after sieving, dropping from 80% (w/w) to about 50% (w/w).

In all cases, drug recovery from Antide–LM (Table 1) was above 85% (w/w) of the nominal content confirming that Antide was not significantly affected by process conditions. The rigid conformation of Antide, stabilized by intramolecular forces, shown in NMR conformational study [25], could explain its high resistance to high temperatures and solvents.

3.3. Lipid microparticles surface morphology by scanning electron microscopy

Fig. 1 shows SEM images of Antide–LM based on Compritol E ATO, prepared, respectively, by

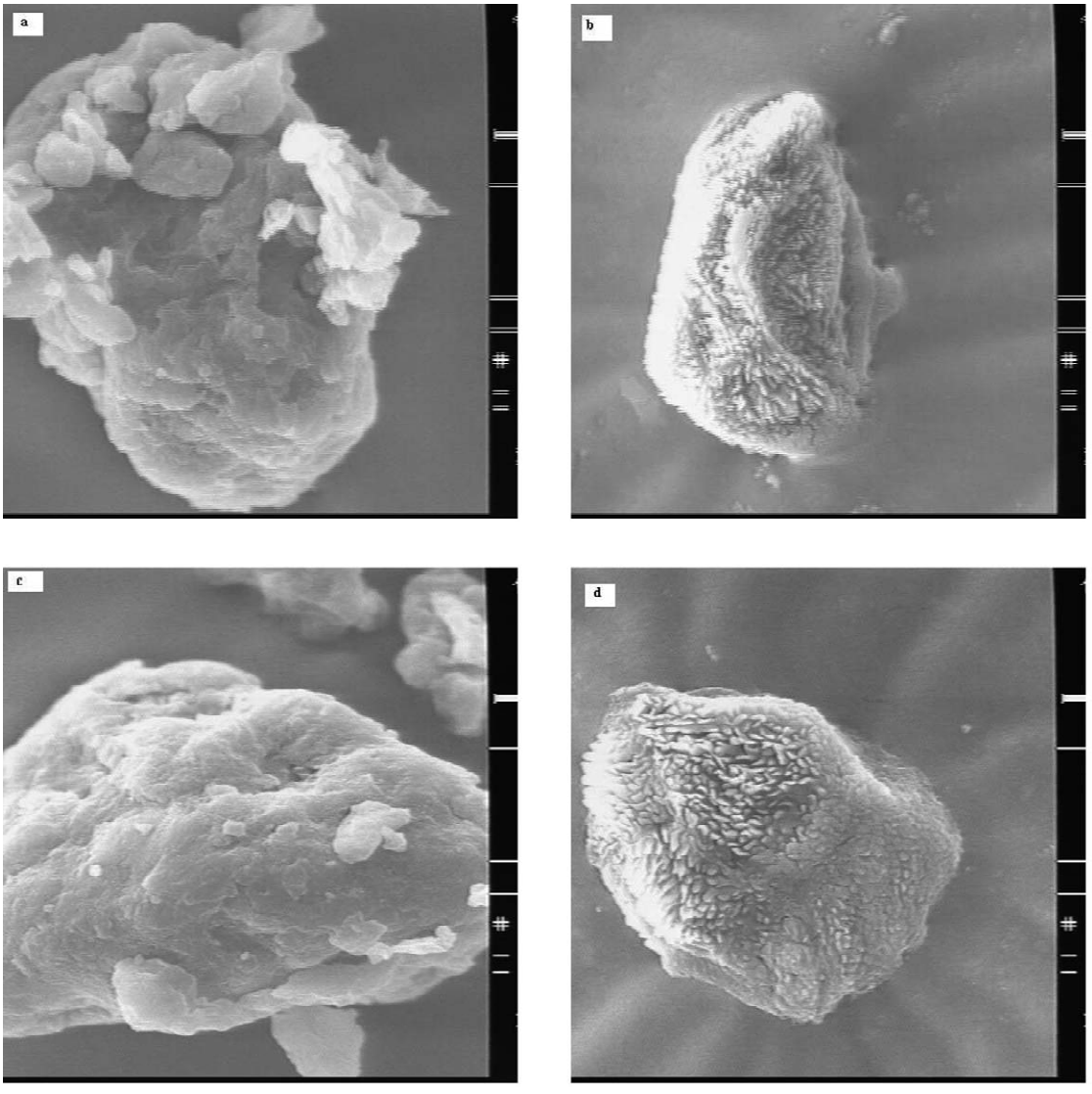

Fig. 1. Scanning electron microscopy of (a) Antide–LMs prepared by co-melting at $t=0$ h; (b) Antide–LMs prepared by co-melting incubated in PBS at 37 °C for 24 h; (c) Antide–LMs prepared by solvent stripping at $t=0$ h; (d) Antide–LMs prepared by solvent stripping incubated in PBS at 37 °C for 24 h (original magnification, 2000×).

co-melting (LM3) and solvent evaporation procedure (LM1), immediately after preparation (Fig. 1a and c) and after 24 h incubation in PBS, pH 7.4 (Fig. 1b and d). LMs immediately after preparation show an irregular shape, nonhomogeneous particle size and a compact and rough surface. When in contact with physiological media for 24 h, the whole surface undergoes the formation of lamellae; no major influence can be ascribed to the preparation procedure (co-melting or solvent stripping), either at $t=0$ or at $t=24$ h. Based on SEM analysis, it can be hypothesized that the release mechanism of the peptide from lipid microparticles is mainly dependent on the surface erosion of the matrix. However, the in vivo fate of lipid microparticles could be significantly different due to possible presence at the injection site of enzymes such as lipases that may degrade the particles, as was shown for solid lipid nanoparticles, incubated with pancreatic lipase in combination with pancreatic co-lipase [26].

3.4. In vitro release study

Several efforts have been made to develop an in vitro release method that could be employed as a rapid quality control test during microparticle development or commercial manufacturing [27]. Furthermore, the correct prediction of in vivo peptide release from microparticles has been attempted and can be affected by the type of release medium [28] and the release method itself [29]. In this study the main objective was to develop a standardized reproducible in vitro release method that could support LM characterization in this and in later production stages. Therefore, an USP apparatus II was chosen and the experiment duration was fixed at 24 h in order to shorten the test and monitor only the initial release phase. Such a time interval was considered sufficient to discriminate among different formulations and predict their sustained release potential. We evaluated a posteriori whether the results obtained from the in vitro release could correlate with the PK profiles obtained in vivo after subcutaneous administration in rats for the time period considered (24 h).

In Fig. 2 the in vitro dissolution profiles from Antide–LM are reported. Different kinetics seem to depend on the incorporation procedure and on the matrix components. Antide–LM prepared by the stripping procedure have a slower release than those prepared by co-melting. The initial burst was below 20% for Compritol E ATO microparticles (5.3% at 6 h) and for Imwitor 900 (18.1% at 6 h). Nevertheless

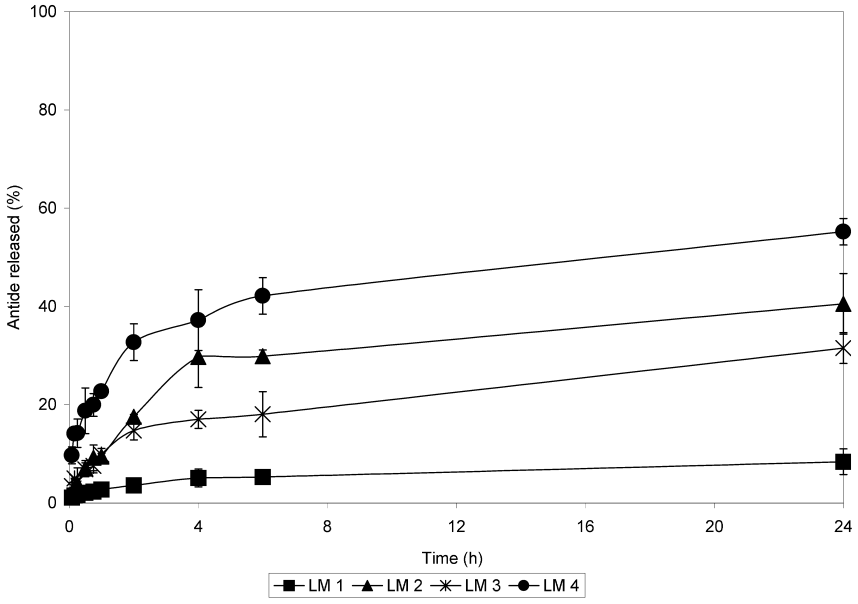

Fig. 2. In vitro cumulative release profiles up to 24 h of 2% (w/w) Antide loaded LM1, LM2, LM3 and LM4 batches. Release conditions: USP dissolution apparatus II, deionized water as release medium, 37 °C, 100 rpm.

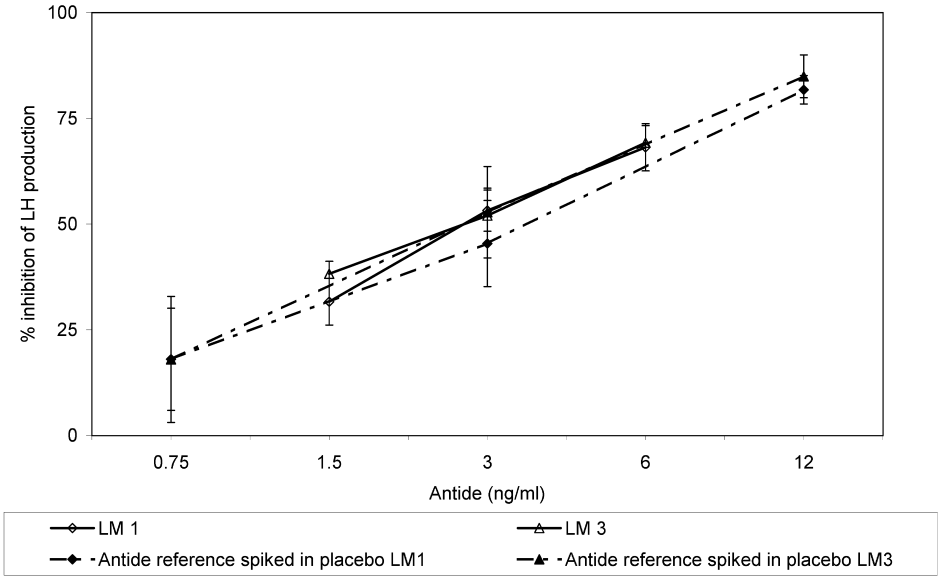

Fig. 3. In vitro bioactivity test results (rat pituitary cell bioassay) of Antide loaded LM1, LM3 batches and their respective placebo spiked with Antide reference.

LMs based on Compritol E ATO show slower release kinetics than those based on Imwitor 900 irrespective of the manufacturing procedure. Antide release was, in the case of co-melting, 29.8 and 42.6%, respectively, after 6 h. SEM analysis did not reveal any difference in the surface structure among LM preparations, either immediately after preparation or after incubation in simulated physiological medium. However, all microparticles obtained by co-melting, both placebo and Antide loaded and for both monoglycerides, are better wetted than those obtained by stripping (contact angle measurements, data not shown), suggesting that the wettability properties of the matrix can influence the initial peptide release. It can be speculated that during the solvent stripping procedure a preferential orientation of the monoglyceryde at liquid–air interface may occur. During the evaporation process the organic solvent might favor the exposure of the lipophilic tail of monoglyceride to the air–liquid interface.

3.5. In vitro bioactivity

In order to assess the activity of the Antide incorporated in the LM formulations, two independent experiments were carried out. As shown in Fig. 3, no main differences were found between Antide extracted from the two LM formulations based on

Table 2
Pharmacokinetic parameters following single subcutaneous administration of Antide–LM batches in rats

Batches	C_{max} (ng ml^{-1})	t_{max} (h)	$t_{1/2}$ (h)	C_n (ng ml^{-1})	AUC $_{(0\rightarrow 24h)}$ (h ng ml^{-1})	AUC$_n$ (h ng ml^{-1})	AUC $_{(0\rightarrow\infty)}$ (h ng ml^{-1})
Antide solution	333.5±113.9	2	18.3	3.9	2426.9±324.4	3294.4	3397.3
LM1	125.1±34.3	2	355.4	3.5	815.6±24.3	4137.2	5931.7
LM2	89.1±13.2	2	53.5	0.5	942.5±93.2	1883.3	1921.9
LM3	178.5±23.5	2	584.8	3.6	1949.8±164.0	6035.8	9073.3
LM4	412.1±40.4	2	161.1	2.1	3051.9±157.5	4907.1	5395.1

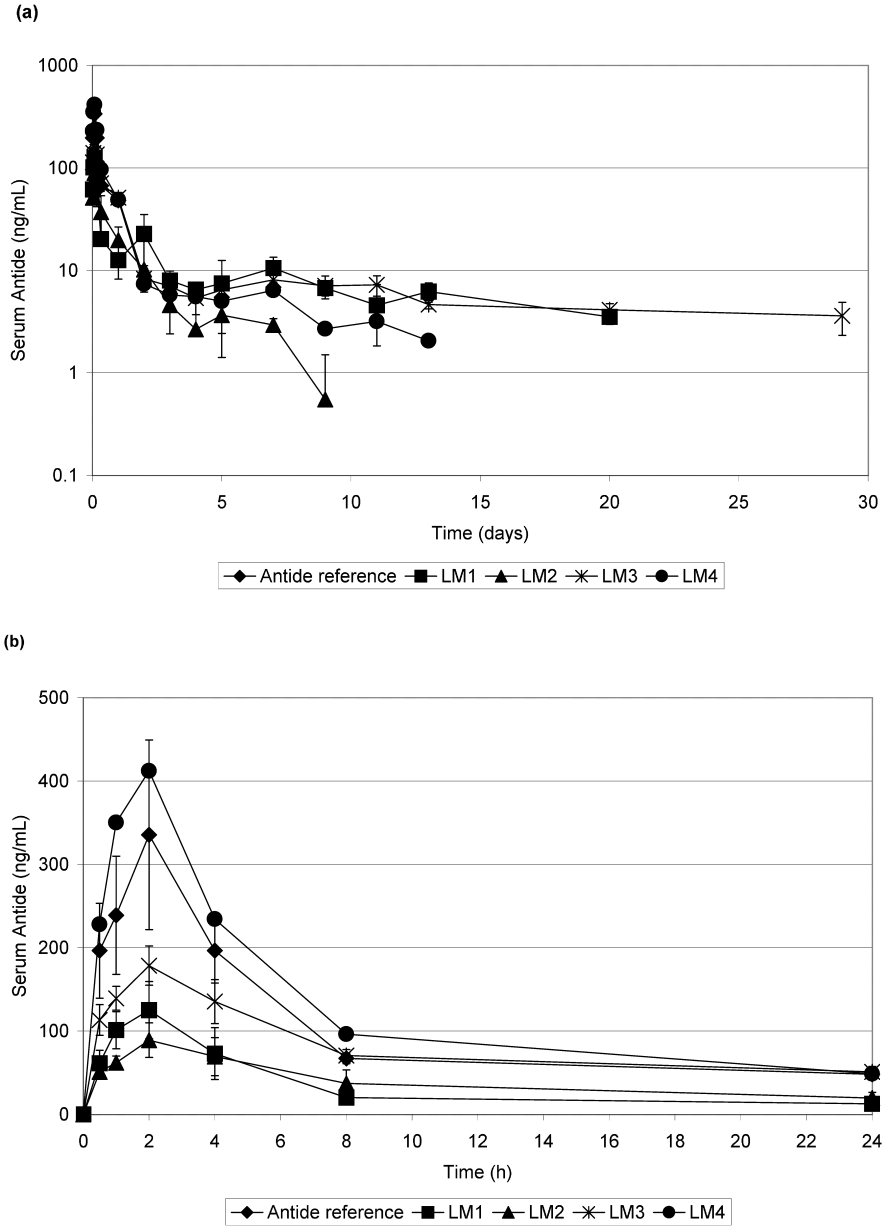

Fig. 4. In vivo serum Antide levels (0 h–30 days (a), 0–24 h, (b, linear scale) after single subcutaneous administration of Antide reference and Antide loaded LM1, LM2, LM3 and LM4 batches (dose 2 mg/rat, $n=3$).

Compritol E ATO and Antide spiked in the relevant placebo. The recovery was almost complete, 105% for LM1 and 109% for LM3, indicating no bioactivity loss.

When Antide reference material was spiked in the culture medium containing the placebo of Imwitor-900-based LM, a lower activity was observed. A similar decrease in bioactivity was also detected with Antide extracted from corresponding loaded microparticles, thus supporting the idea that the Imwitor 900 might interfere with the assay (data not shown).

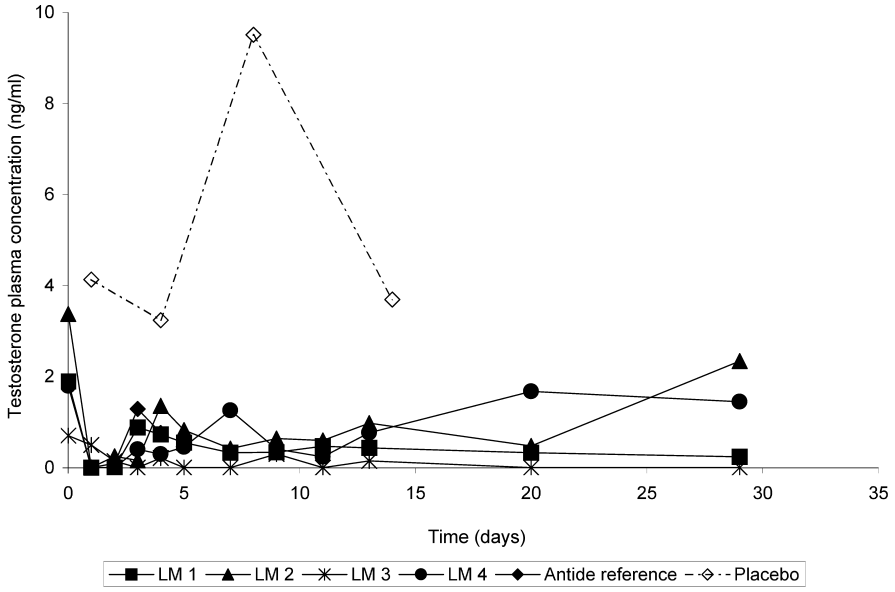

Fig. 5. In vivo testosterone levels after single subcutaneous administration of Antide reference and Antide loaded LM1, LM2, LM3 and LM4 batches (dose 2 mg/rat, $n=3$).

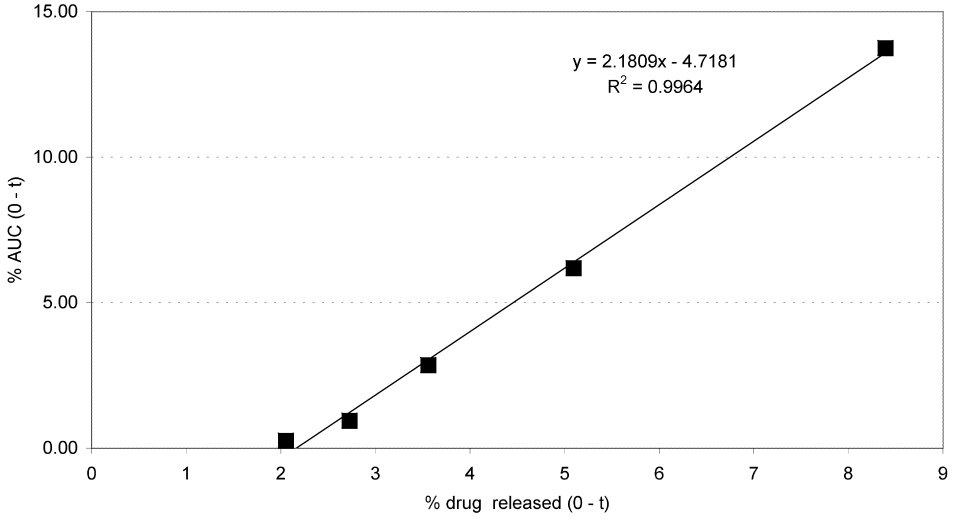

Fig. 6. In vitro–in vivo correlation profiles of Antide loaded LM1 batch, plotted as AUC at different times up to 24 h vs. corresponding in vitro percentage Antide released.

3.6. In vivo antide pharmacokinetic and pharmacodynamic profiles

The main pharmacokinetic parameters calculated from the mean plasma profiles of Antide LM according to a noncompartimental analysis, are summarized in Table 2. The mean plasma profiles of Antide–LMs are plotted in Fig. 4.

In the reference group receiving Antide solution, mean plasma concentrations gradually increased up

to 2 h ($C_{max} = 333.5$ ng/ml). The levels then declined to 48 ng/ml at 24 h followed by a further decline to 7.3 ng/ml at 48 h and 2.8 ng/ml at 7.2 h. The half-life was about 18.3 h.

The pharmacokinetic profiles of LMs were also characterized by a t_{max} of 2 h, while $t_{1/2}$ was considerably increased for all LM considered, reaching up to 350 and 584 h for LM1 and LM3, respectively, and 53 and 161 h for LM2 and LM4.

Mean C_{max} varied from 89±21 ng/ml for LM2 to 412±42 ng/ml for LM4. For LM 1 and LM3 the C_{max} values were 125±34 and 179±24 ng/ml. Concerning the duration of release, the best results were obtained for LM3, with quantifiable plasma levels up to 29 days. LM1 reached 20 days, LM 2 reached 9 days while LM 4 reached 13 days. The initial burst can be effectively minimised in LM formulations as observed in the initial pharmacokinetic profile (0–24 h), represented on a linear scale (Fig. 4b). In conclusion, considering the complete period of observation (0–29 days) from a pharmacokinetic point of view, LM3 produced the best releasing profile of Antide, with a limited initial burst and the longest release period.

Testosterone plasma concentrations are reported in Fig. 5. The suppression of testosterone following administration of Antide–LMs was assessed by comparing the hormone levels detected at the different times with those measured before treatment (time 0) and/or in comparison with the testosterone levels observed in the placebo group (ranging from 3.2 to 9.5 ng/ml, mean values) and with the group treated with Antide solution. In this latter group suppression of testosterone levels lasted for 2 days. On day 3 a mean level of 1.3±0.2 ng/ml was observed, reflecting a return to normality. In all LM-treated groups hormone levels lower than those measured in untreated animals were found (at least up to 13 days from treatment). A real suppression, with testosterone levels below LOQ (0.2 ng/ml) up to the last observation time (29 days), was found only in almost all animals treated with LM1 and LM3.

Such observations are in good agreement with the measured pharmacokinetics, confirming that both lipid matrix components (stearate or behenate) and incorporation method (solvent stripping or co-melting) play a role in controlling Antide release from microparticles. However, from the in vivo data it could be assumed that the characteristics of the matrix are more influential than the incorporation procedure (see also PK profiles of LM1 and LM4).

An attempt to correlate the in vitro and the in vivo release was made, by plotting the AUC values as a function of the percentage drug released in vitro, at

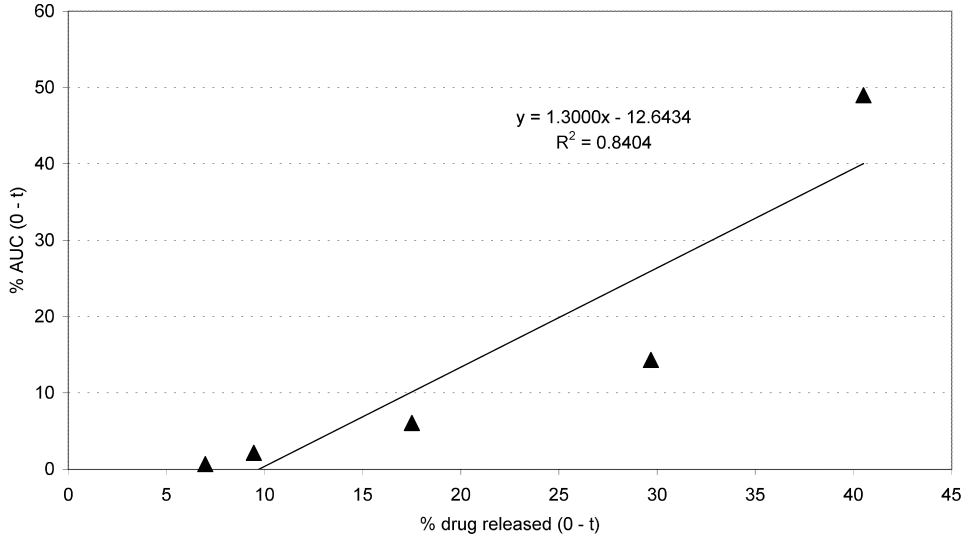

Fig. 7. In vitro–in vivo correlation profiles of Antide loaded LM2 batch, plotted as AUC at different times up to 24 h vs. corresponding in vitro percentage Antide released.

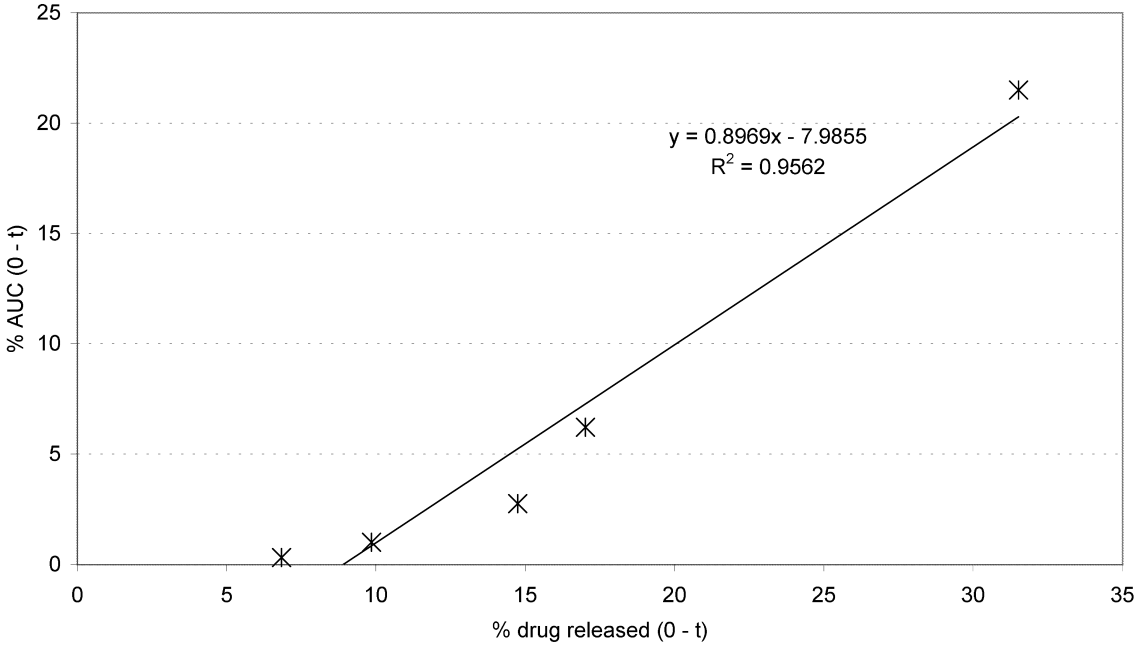

Fig. 8. In vitro–in vivo correlation profiles of Antide loaded LM3 batch, plotted as AUC at different times up to 24 h vs. corresponding in vitro percentage Antide released.

corresponding time points within the time interval considered (0–24 h). Figs. 6–9 report the relevant curves obtained for LM formulations. A good correlation was observed for LM 1 ($R=0.996$), LM3 (0.956), LM4 (0.954), while a poor correlation was shown for LM2 (0.840). (This latter value could be

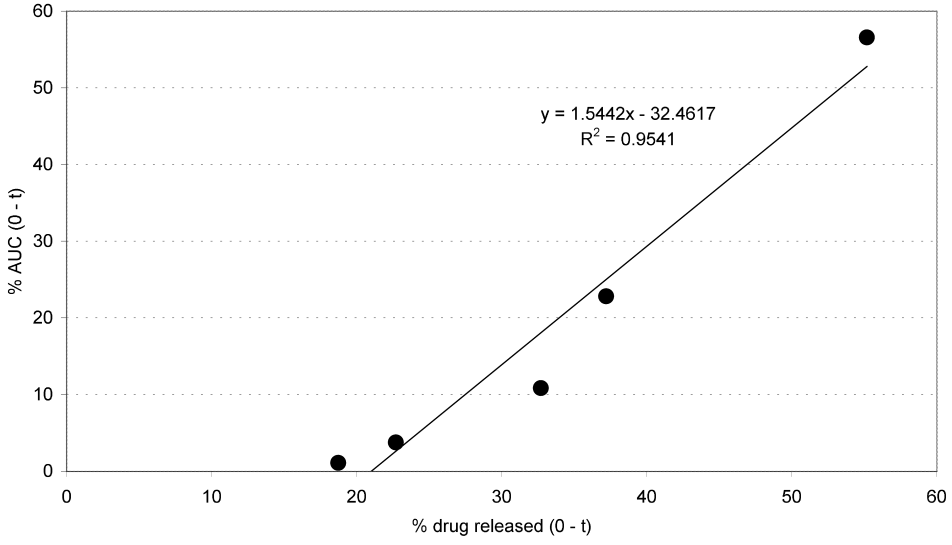

Fig. 9. In vitro–in vivo correlation profiles of Antide loaded LM4 batch, plotted as AUC at different times up to 24 h vs. corresponding in vitro percentage Antide released.

due to the problems which occurred during the in vivo trials that hampered a complete administration of the theoretical dose). These results show that the in vitro release established in this study was successful in predicting the in vivo release potential of LM formulations and could be taken into consideration in the future as possible screening method for the formulation development.

4. Conclusions

Lipid microparticles loaded with a GnRH antagonist (Antide), suitable for s.c. and i.m injection, have been successfully prepared by cryogenic micronization and characterized. The in vitro biological activity of Antide was not affected by the process conditions. Lipid microparticles provided an in vivo (rats) sustained release of Antide for 1 month with a parallel complete testosterone suppression in the case of Antide–LM, based on Compritol E ATO and obtained by co-melting procedure. The levels of initial drug release can be influenced by lipid characteristics and by process conditions. In vitro release of Antide correlated well with the in vivo release in almost all Antide–LM batches considered. Antide–LM are potentially useful as depot formulation in all the clinical indications where a long testosterone suppression is required.

Acknowledgements

The authors thank Flavio Peroglio (LCG-RBM, Colleretto Giacosa, Turin, Italy) for SEM analyses.

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