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Long-acting delivery systems for peptides: reduced plasma testosterone levels in male rats after a single injection

M.R. Gasco [1], F. Pattarino [1] and F. Lattanzi [2]

[1] *Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi, Torino (Italy) and* [2] *SCLAVO, Siena (Italy)*

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Summary

The study of a biocompatible w/o microemulsion for parenteral administration of peptides was performed. The behaviour of an LH-RH analog, [D-Trp-6]LH-RH, in a w/o microemulsion was evaluated both in vitro and in vivo. After a single intramuscular injection in rats of the microemulsion containing 500 μg/ml of the drug, the plasma levels of testosterone fell; between 10 and 20 days after injection, the plasma testosterone level was lower than that observed after daily injections of 100 μg/kg of the LH-RH analog. The results suggested that prolonged action and protection of biodegradable molecules could be achieved by using w/o microemulsions administered parenterally.

Introduction

Luteinizing hormone-releasing hormone (LH-RH) activates the release of the pituitary hormones which control reproductive development. Several LH-RH analogs were synthesized and tested for both in vitro and in vivo activity; when the sixth amino acid was replaced with D-amino acids, such as [D-Trp-6]LH-RH, the analogs demonstrated greater activity than the parent molecule (Coy et al., 1976).

Although [D-Trp-6]LH-RH has a half-life longer than that of LH-RH (2 h compared to 15 min), prolonged delivery systems could offer the advantage of maintaining controlled levels of peptide over an extended period of time, thus reducing the frequency of administration. The incorporation of [D-Trp-6]LH-RH into microcapsules of poly-(DL-lactide–co-glycolide) (Redding et al., 1984; Mason-Garcia et al., 1985, Schally and Redding, 1985) can represent a convenient and powerful approach.

The aim of the present work was to achieve prolonged action of the LH-RH analog by using a liquid delivery system such as a w/o microemulsion.

Experimental

Materials

Egg lecithin, chloroform, methanol and Silica Gel were obtained from Merck, ethyl oleate from Carlo Erba, caproic acid from Fluka, and phospholipids for use as standards were Sigma prod-

Correspondence: M.R. Gasco, Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi, Torino, Italy.

ucts. [D-Trp-6]LH-RH was purchased from Sclavo (Siena).

Instruments

An Ubbelhode viscometer (Scott-Geräte), Zetasizer 2c (Malvern Instruments), centrifuge 4225 (ALC) and Biofuge A (Heraeus Christ) were used.

The chromatographic system consisted of a 2010 isocratic pump, a 2050 variable UV detector, a 7125 Rheodyne injector and a 4290 integrator (all from Varian).

Preparation of w/o microemulsion

The studied w/o microemulsion was prepared as described previously (Gallarate et al., 1988), using ethyl oleate (60.5%), water (10.1%), phosphatidylcholine (PC) (18.9%) and caproic acid (10.5%).

The natural surface-active agent, PC, was obtained by flash chromatography of a phospholipid mixture (Gallarate et al., 1988) on a silica gel column eluted with chloroform/methanol/water (60:30:4, v/v). PC was stored at −20 °C in acetone until use.

In order to obtain microemulsions containing drug, weighed amounts of [D-Trp-6]LH-RH were dissolved in the water phase before preparation.

Viscosity

Viscosity measurements on the microemulsion were performed using a thermostated capillary viscometer at both 25 and 37 °C. Microemulsions with or without drug were tested.

Diameter of disperse phase

Light-scattering measurement allowed us to determine the particle size of the w/o microemulsion being studied through the evaluation of the diffusion coefficients of the dispersed droplets undergoing Brownian motion.

The mean diameter of particles was evaluated through a simple Stokes-Einstein formula.

Analysis

The chromatographic method proposed by Sertl et al. (1981) was modified in order to allow the analysis of LH-RH analog samples containing components from the microemulsion. Various compositions of the mobile phase (phosphate buffer pH 6.5/acetonitrile, 70:30–80:20) and flow rates (1.0–1.5 ml/min) were used.

The purity of PC was tested by HPLC according to Carunchio et al. (1984).

Degassing of the solvents was achieved by filtration and sonication; all samples were filtered through a 0.22 μm Millex GV4 filter before injection.

In vitro diffusion The ability of the disperse phase of the microemulsion to provide prolonged release was assessed by investigation of diffusion.

The diffusion experiments were carried out using as donor phase: (1) a buffered solution; (2) the w/o microemulsion; (3) mixed systems comprising microemulsion and phosphate buffer at several ratios (1:9, 1:1.5, 1:0.67, v/v).

The final drug concentration of [D-Trp-6]LH-RH in the donor compartment at time = 0, was always 500 μg/ml; a phosphate solution (0.025 M; pH 6.5) was used as acceptor phase. In the diffusion experiments, sink conditions were achieved by using a cell constituted of two compartments of different capacity (10 ml donor and 175 ml receiving volumes, respectively); the compartments were separated by a Servapor dialysis membrane (cut-off = 12 000 Da), wetted with the receiving phase. The cell was warmed in a jacketed container maintained at 25 °C and the cell contents were stirred by bar-shaped magnets rotating at about 300 rpm. Aliquots of 200 μl of acceptor phase were removed at fixed times and analyzed.

The transfer of the drug from a buffered solution to the microemulsion was also examined. The diffusion of [D-Trp-6]LH-RH from the solution to the microemulsion was performed by using a 2 mg/ml drug phosphate solution as donor phase; microemulsion represented the receiving phase. A glass apparatus, similar to that described by Nakano and Patel (1970), was utilized in these experiments. Membrane, temperature control, stirring conditions and analytical methods were the same as for the diffusion experiments.

In vivo experiments The biological effects of [D-Trp-6]LH-RH, following the administration of microemulsion and buffered solution, respectively, were evaluated in rats.

Adult male Sprague-Dawley rats, weighing approx. 200 g, maintained under controlled conditions of light (12 h dark/12 h light cycle) and temperature (22° C) were used. 28 randomized rats were treated with 3 mg/kg i.m. of the drug dispersed in freshly prepared microemulsion. A second group composed of 28 animals was injected i.m. with 100 μg/kg of LH-RH analog in buffered solution each day for 28 days. At scheduled times (1, 3, 5, 7, 14, 21, 28 days) blood samples from four rats in each group and from two controls were collected and plasma was immediately separated by centrifugation and stored at −20° C until analyzed. Plasma was assayed for testosterone using a commercially available RIA kit (Radioassay Systems Laboratories).

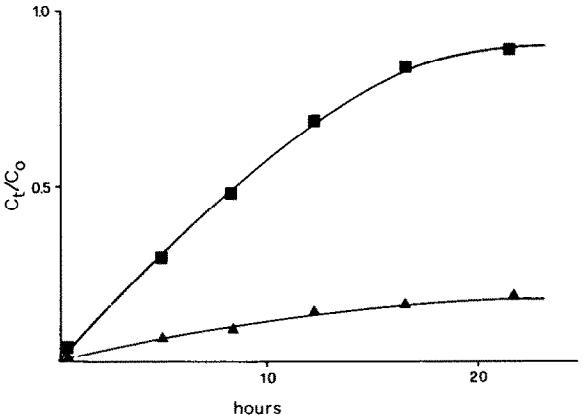

Fig. 1. The in vitro diffusion of [D-Trp-6]LH-RH from buffered solution (■) and 1:9 microemulsion/solution mixture (▲); diffusion peptide fraction (C_t/C_0) vs time (h).

Results

Viscosity

Viscosity measurements of the w/o microemulsion in the presence and absence of the drug gave the same values (21.8 cSt at 25° C and 16.23 cSt at 37° C, respectively).

Particle size

By using the QELS technique, the mean diameter of the dispersed nanodroplets was found to be 11.6 nm (polydispersity = 0.607). The presence of the LH-RH agonist in the microemulsion had no significant effect on this result.

HPLC analysis

In the [D-Trp-6]LH-RH analysis, linearity was observed between 8.0 and 170 μg/ml ($r > 0.9980$) as expected. The resolution improved on changing both the composition and flow rate of the eluent.

The surfactant analysis revealed the presence of PC (> 97.0%) and of small amounts of PS, PE and PI.

Diffusion studies

The appearance of LH-RH analog in the acceptor compartments was monitored as a function of time (Fig. 1). No appreciable release of [D-Trp-6]LH-RH from the microemulsion could be noted within 24 h. Also, from two microemulsion/aqueous solution mixtures (1:1.5 and 1:0.67) no diffusion occurred, whereas from the 1:9 mixture significant release was observed. However, the amounts of diffused drug were less than those observed for the [D-Trp-6]LH-RH solution.

In the transfer experiments, no appreciable diffusion of drug from solution to microemulsion was observed.

Plasma testosterone levels after i.m. administration of microemulsion containing LH-RH analog

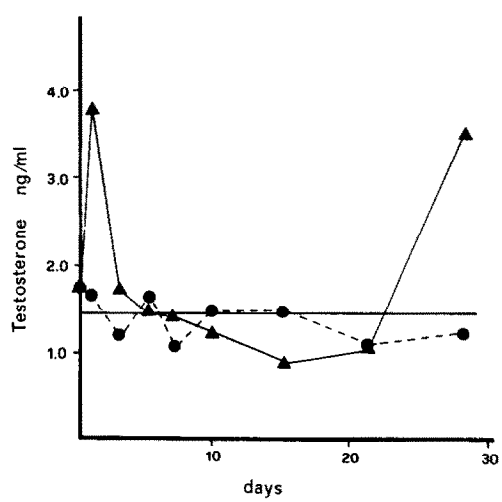

Fig. 2. Plot of mean plasma concentration-time curves of testosterone in rats, following a single i.m. injection (3 mg/kg) of [D-Trp-6]LH-RH in microemulsion (▲) and daily i.m. injection (100 μg/kg) of buffered solution (●).

showed the following profile (Fig. 2):
(a) significant increase in plasma levels 24 h after the injection, returning to normal concentrations within 3 days;
(b) testosterone levels slightly lower than those of controls between the 4th and 21st days;
(c) testosterone concentration higher than those of the controls after 28 days.

A constant depression of plasma gonadal hormone levels was shown by the daily administration of [D-Trp-6]LH-RH solution.

Discussion

Many authors (Mason-Garcia et al., 1985; Schally and Redding, 1985) have studied the effect of subcutaneous injections of LH-RH agonists in biodegradable microcapsules, observing marked biological effects in rats for several weeks. The microcapsules of biodegradable esters were prepared by phase separation with halogenated solvents which could not be removed completely; moreover, there were differences between different batches of microcapsules.

Thus, the development of alternative long-acting delivery systems for the parenteral administration of LH-RH analogs is of considerable interest. Microemulsions appear to possess interesting properties which are potentially usable for prolonged release: they are clear systems at infinite stability, reproducible and readily prepared, and their disperse phase can act as a reservoir of drugs (Rand, 1976).

In this study, we used a w/o microemulsion composed of natural and biocompatible compounds. The dispersed nanodroplets had a mean diameter close to that of the swollen micelles and the addition of agonist did not affect this value. The [D-Trp-6]LH-RH, added on the formation of the microemulsion or at the aqueous phase before preparation of the microemulsion, dissolved in the internal phase.

The in vitro experiments suggested that our w/o microemulsion could be utilized for the controlled release of LH-RH analog over a few weeks. The lack of diffusion of [D-Trp-6]LH-RH from the microemulsion indicated that the peptide, under the present experimental conditions, was partially protected from the environment. This behaviour was emphasized by the lack of permeation of the drug from the solution to the microemulsion.

These results prompted us to undertake the in vivo experiments. Our formulation, injected into rats, yielded low levels of plasma testosterone (Fig. 2). By using the microemulsion, the profile of testosterone concentrations that resulted was similar to that of other sustained release formulations (Mason-Garcia et al., 1985). Gonadal hormone levels, after administration of the microemulsion system, were comparable to those achieved by daily injections. In both cases, the testosterone concentrations were lower than those for control rats. The suppression of plasma testosterone concentration in rats was maintained over 3 weeks.

The w/o microemulsion administered by the parenteral route shows some advantages: small volumes of the injection, ease and reproducibility of the preparation, infinite stability of the system, biocompatibility of all the components, and partial protection of biodegradable molecules by the environment.

The results of this preliminary study indicate the utility of w/o microemulsions for the administration of biodegradable molecules. Further studies are in progress to improve and to optimize the release of peptides from microemulsions.

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