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Radioimmunoassay of [D-Trp^6]-luteinizing hormone-releasing hormone: its application to animal pharmacokinetic studies after single injection and long-acting formulation administration

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Summary

A sensitive radioimmunoassay (RIA) for [D-Trp^6]-luteinizing hormone-releasing hormone (LHRH) has been developed. This assay allowed measurement of the LHRH analog in unextracted plasma with a minimum detectable concentration of 10 pg/ml. Validation of plasma assays was performed through Sep-Pak and HPLC purification. The in vivo fate of the peptide was investigated in dogs after subcutaneous or intravenous injections. In both cases, the LHRH analog showed longer plasma half-life than native LHRH with an elimination half-life superior to 80 min. Long-acting formulations were tested in dogs and rats: the day following administration, [D-Trp^6]-LHRH plasma level rose to 2.9–4.6 ng/ml in dogs and 0.8–3.8 ng/ml in rats. From day 4 to day 30, [D-Trp^6]-LHRH plasma level followed a plateau with concentrations of 0.3–0.8 ng/ml in dogs and 0.2–0.4 ng/ml in rats. In parallel, testosterone plasma concentration was reduced to castrate level between day 4 and day 7 in dogs and was significantly lowered in rats. This sensitive [D-Trp^6]-LHRH RIA will be particularly useful for the evaluation of long-acting formulations in patients with advanced prostate cancer.

radioimmunoassay; LHRH; pharmacokinetics; long-acting formulations; prostate cancer

Introduction

Recent findings have suggested that regression of various hormone-dependent disorders and, mainly, prostate tumors can be achieved after administration of LHRH agonists [1–5]. These substances, chronically administered, induce a strong reduction of both gonadotropin and sexual steroids secretions [6,7]. This biological effect is the result of their higher resistance to proteolytic breakdown and/or increased affinity to pituitary or peripheral receptors [8,9]. LHRH analogs with modification at the 6-position show lower degradability than does native LHRH after incubation with tissue homogenates [10,11]. Among these analogs [D-Trp^6]-LHRH has been widely used and its biological and clinical effects are well documented [3]. Although expected effects are obtained with daily injections of [D-Trp^6]-LHRH, a long-acting release formulation, consisting of biodegradable microcapsules, is a more suitable mode of administration [12]. In this case, the assessment of the release and the blood level of the peptide is of particular importance to assure the desired biological effects. For these reasons, a specific and sensitive immunoassay should be used. In this paper, we describe a radioimmunoassay for this analog in unextracted plasma and its application to pharmacokinetic studies and to the optimization of long-acting formulations.

Material and Methods

Preparation of [D-Trp^6]-LHRH conjugates and immunization procedure

Two immunogens were prepared by coupling the peptide to bovine serum albumin (BSA) (Boehringer) with two different agents, glutaraldehyde or benzoquinone.

In the first case, [D-Trp^6]-LHRH was dissolved in 0.1 M phosphate buffer saline (pH 7.4) to a concentration of 20 mg/ml. 0.5 ml of the solution was mixed with BSA (10 mg in 0.15 M NaCl) and glutaraldehyde (Eastman) (0.2 ml at 0.5%). The mixture was allowed to react 24 h at room temperature and then subjected to AcA 202 gel filtration (Ultrogel) and eluted with 0.15 M NaCl.

In the second case, BSA was at first activated with 0.3 ml benzoquinone (Fluka AG) (30 mg/ml in ethanol). Excess benzoquinone was removed through an AcA 202 column. 1 mg of [D-Trp^6]-LHRH in 1 ml bicarbonate buffer (0.5 M), pH 9.5, was added to 1 ml of BSA-benzoquinone derivative. Coupling was performed at room temperature in darkness during 24 h. Uncoupled peptide was eliminated through gel filtration (AcA 202).

In each case, the incorporation of 100 000 cpm of iodinated [D-Trp^6]-LHRH in the reaction mixture allowed us to measure the yield of the coupling reaction. According to the radioactivity eluted in the void volume, we found 2.8 (with glutaraldehyde) and 6.1 (with benzoquinone) molecules of [D-Trp^6]-LHRH coupled per molecule of BSA. Each immunogen emulsified in Freund's complete adjuvant (Difco) was injected intradermally at multiple sites in three adult male rabbits. Animals received approximately an equivalent of 100 μg of [D-Trp^6]-LHRH at the first injection and 10–50 μg at booster injections. Rabbits were bled from the central ear artery at various times. Sera were stored at -20°C in 0.01% NaN_3 and 50% glycerol.

Iodination of [D-Trp^6]-LHRH and purification of labelled hormone

The peptide was radioiodinated according to the iodogen method [13]. Iodogen (Pierce), 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (1 μg in 10 μl dichloromethane), was added to a plastic tube (Eppendorf). The organic solvent was evaporated under N_2. Then 25 μl phosphate buffer (0.5 M), pH 7.4, 5 μl acetic acid (0.1 N) solution of [D-Trp^6]-LHRH (2.5 pg or 2 nmol) and 5 μl of Na^{125}I (≅ 750 μCi, Radiochemical Center, Amersham) were successively added. 7 min later, the reaction mixture was chromatographed on carboxymethyl cellulose (CMC 52, Whatman). Free iodide was eluted with 0.005 M ammonium acetate buffer, pH 4.7. The column was then washed with 30 ml ammonium acetate buffer (0.025 M), pH 4.7. Iodinated [D-Trp^6]-LHRH was eluted with 0.100 M and 0.150 M ammonium acetate buffer, pH 4.7, and collected in 0.5 ml PBS-BSA. Immunoreactivity was calculated by incubating [^{125}I]-[D-Trp^6]-LHRH with an excess of antiserum. The tracer was aliquoted and stored at −80°C.

[D-Trp^6]-LHRH radioimmunoassay

The diluent used in binding experiments and RIA for [D-Trp^6]-LHRH was 0.05 M phosphate buffer, pH 7.4, containing 0.5% BSA and 0.15 M NaCl. RIA was performed in polystyrene tubes using a total volume of 0.3 ml. The standard was dissolved in 0.1 M acetic acid, aliquoted and stored at −80°C. Standard or sample (0.1 ml) and diluted [D-Trp^6]-LHRH antiserum (0.1 ml) were first preincubated for 24 h at 4°C. Then [^{125}I-D-Trp^6]-LHRH (≅ 10 000 cpm, 0.1 ml) was added and incubated again 24 h at 4°C. The antigen-antibody complex was separated by adding 10 μl normal rabbit serum and 1 ml ice-cold propanol-1. The tubes were then centrifuged at 3500 rpm for 15 min at 4°C and the supernatants aspirated. The radioactivity of the precipitate was measured in a gammacounter (Nuclear Enterprises 1600). All samples were assayed in duplicate.

Validation of plasma assay

Sample purification. A Sep-Pak reversed C18 (Waters Associates) was used to extract the peptide from plasma samples. The top of the column was attached to a 3-ml disposable plastic syringe. A volume of 0.5 ml of plasma was applied to the Sep-Pak cartridge previously activated by successive applications of 3 ml methanol and 5 ml distilled water. The column was then washed with 5 ml trifluoroacetic acid (0.1%; TFA, Fluka). The peptide adsorbed on the silica matrix surface was eluted with 5 ml CH_3OH/H_2O/TFA (80:19:1). The eluate was evaporated. The aqueous phase was then frozen and lyophilized samples were reconstituted in the appropriate buffer before radioimmunoassay.

High-performance liquid chromatography (HPLC). For some plasma samples a HPLC step was introduced after the Sep-Pak column. The dried eluate was dissolved in 0.3 ml TFA (0.1%, v/v). A volume of 0.1 ml was injected into a μ-Bondapak C18 column and eluted (1 ml/min) with a linear gradient (Waters Solvent Programmer No. 6) of 5–95% B of the following solvent: solvent A, 11 mM TFA, 3 mM acetic acid; solvent B, 11 mM TFA in 70% acetonitrile. Column eluates were collected in polystyrene tubes with a Gilson 201 fraction collector at 0.5 min intervals (0.5 ml

volume). The fractions were then lyophilized and reconstituted with the appropriate buffer before RIA. Calibrations were performed in running [D-Trp^6]-LHRH after dissolution in TFA (50 μg in 0.1 ml 11 mM TFA) or after Sep-Pak extraction from plasma. Absorbance at 210 nm was monitored with a LC spectrophotometer Model 481 (Waters).

Handling of samples

Blood was collected in heparinized tubes containing aprotinin (1000 KIU/ml) to prevent any enzymatic degradation, chilled, centrifuged and stored at −20°C until radioimmunoassay for [D-Trp^6]-LHRH (with antiserum 2050-11) and testosterone (Sorin Biomedica Testosterone Kit).

Kinetic studies

Single injection studies. Two male Beagle dogs (A1 and B1), weighing respectively 14 and 14.6 kg, were given a bolus intravenous injection of 100 μg [D-Trp^6]-LHRH dissolved in 1 ml saline. Blood was withdrawn just before injection and 1, 5, 10, 20, 40, 60, 75, 90, 120, 135, 150, 180, 240 min after administration.

A series of 6 dogs (A2 to F2) weighing 12–16.8 kg were given a single subcutaneous injection of 100 μg [D-Trp^6]-LHRH which corresponded to an effective dose of 6–8.3 μg/kg. Samples were collected every 15 min the first hour and 2, 3, 4, 6, 8, 12, 24 h after administration.

Long-acting formulations

A long-acting delivery system has been developed and optimized recently: it consists of biodegradable microcapsules of a (±-lactide-coglycolide) copolymer containing 3 mg of available [D-Trp^6]-LHRH to be released within 30 days. Dogs A2 to F2 received an intramuscular injection of one dose of microcapsules (3 mg of [D-Trp^6]-LHRH) in suspension (Cytotech batch 84-27). Blood was withdrawn before treatment and 1, 4, 7, 11, 14, 18, 21, 24, 28, 31 days after injection.

To assess the quality of microcapsules, use of rats seemed more convenient. Long-acting formulations were administered in a volume of 0.15 ml to male Sprague–Dawley rats weighing approximately 300 g. Blood was withdrawn before treatment and 1, 4, 7, 11, 15, 18, 22, 25, 29, 32, 37 days after injection, by retro-orbital puncture.

Results

Production of antibodies

One rabbit was selected from each series which received either immunogen, [D-Trp^6]-LHRH-glutaraldehyde-BSA or [D-Trp^6]-LHRH-benzoquinone-BSA. Corresponding antisera were 2050-11 and 2069-12, respectively.

Specificity was tested with various peptides and LHRH fragments (Table I). Antiserum 2069-12 did not cross-react with LHRH and other peptides. However, antiserum 2050-11 did cross-react with native LHRH and LHRH fragments containing the three amino acids of the terminal part, Arg-Pro-Gly-NH_2. Affinity constants,

TABLE I

Cross-reaction of LHRH fragments and other peptides with [D-Trp^6]-LHRH antiserum

	% Cross-reaction	
	Antiserum 2050-11	Antiserum 2069-12
[D-Trp^6]-LHRH	100	100
LHRH	20	<0.01
[1–5]-LHRH	<0.02	<0.02
[1–7]-LHRH	<0.02	<0.02
[1–8]-LHRH	<0.02	<0.02
[1–9]-LHRH	<0.02	<0.02
[3–10]-LHRH	17	<0.02
[4–10]-LHRH	7	<0.02
[5–10]-LHRH	13	<0.02
[7–10]-LHRH	17	<0.02
Corticotropin-releasing factor	<0.01	<0.05
Thyrotropin-releasing hormone	<0.01	<0.002
Somatostatin	<0.005	<0.02
Substance P	<0.001	<0.001

derived from Scatchard plots [14] for the 2050-11 and 2069-12 antisera were $4 \cdot 10^{11}$ and $0.5 \cdot 10^{11}$ M^{-1}, respectively.

Iodination of [D-Trp^6]-LHRH

Elution with 0.100 M and then 0.150 M ammonium acetate buffer, pH 4.7, produced one peak, respectively. Binding of the radioactivity contained in both these peaks by excess antiserum (final dilution 1:300) was 50–60% and 85–95%, respectively. The first peak might correspond to di-iodinated peptide or degraded product resulting from iodination. Radioactivity from the second peak, corresponding to the monoiodinated peptide was aliquoted, stored at −80°C and used in the RIA. The specific activity was 1150–1300 Ci/g.

Sensitivity of the RIA

Optimization of RIA conditions was performed in order to reach a maximum of sensitivity with both antisera. Displacement of [^{125}I]-[D-Trp^6]-LHRH bound to the antibody by unlabelled [D-Trp^6]-LHRH is shown in Fig. 1. The 50% displacement of radioligand was 4 ± 0.3 and 23 ± 3 pg/tube (mean ± S.D., n = 7) for antisera 2050-11 and 2069-12 (final dilution 1/4 500 and 1/10 000) with an initial binding of 30%.

Validation of plasma assay

Direct assay of unextracted or undiluted plasma free of [D-Trp^6]-LHRH showed the existence of non-specific response leading to apparent level of immunoassayable

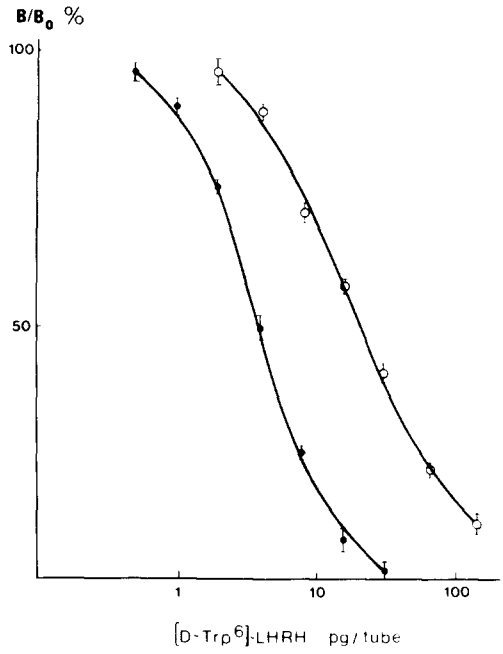

Fig. 1. Displacement of [^{125}I]-[D-Trp^6]-LHRH bound to antisera 2050-11 (●) and 2069-12 (○) by increasing concentrations of [D-Trp^6]-LHRH. B and B_0 represent bound tracer with and without unlabelled ligand, respectively. Each point represents the mean ± S.D. of duplicates.

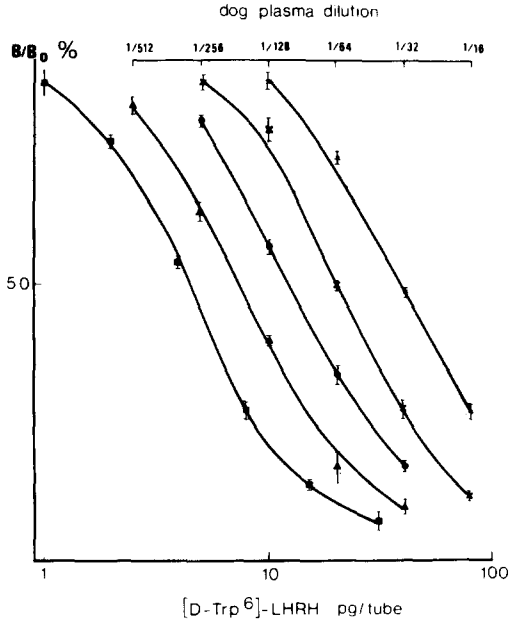

Fig. 2. Parallelism between [D-Trp^6]-LHRH standard curve (■) (antiserum 2050-11) and serial dilutions of dog samples collected at 20 min (▲), 40 min (●), 75 min (×) and 150 min (*) after intravenous injection. Each point represents the mean ± S.D. of duplicates.

[D-Trp^6]-LHRH activity. However this level was lower with antiserum 2050-11 (< 0.15 ng/ml) than with antiserum 2069-12 (0.5–1.5 ng/ml). We determined that the substances which reduce the binding of [^{125}I-D-Trp^6]-LHRH with antiserum 2069-12 were high molecular weight substances (M_r > 60 000) (data not shown). RIA performed with the antiserum 2050-11 was sensitive enough to reduce these interferences. Diluted samples from intravenous injection showed good parallelism with the standard curve (Fig. 2). Therefore antiserum 2050-11 was used in all direct [D-Trp^6]-LHRH assays. Immunoreactive material was identified by Sep-Pak and HPLC purifications as follows: samples from intravenous injection were Sep-Pak extracted and submitted to HPLC. Fractionation produced a single peak which co-eluted exclusively with synthetic [D-Trp^6]-LHRH (Fig. 3). The recovery of [D-Trp^6]-LHRH (30–2000 pg) added to plasma was 80 ± 8% (mean ± S.D., n = 8) after Sep-Pak purification and 63% after HPLC.

Reproducibility

Intra assay coefficient was estimated by determining 30 times [D-Trp^6]-LHRH level in one sample in the same assay; coefficient was 8.3%. Between assay reproducibility was 9.1%, calculated after assaying 10 samples (concentrations ranging from 2 to 25 ng/ml) at three months interval.

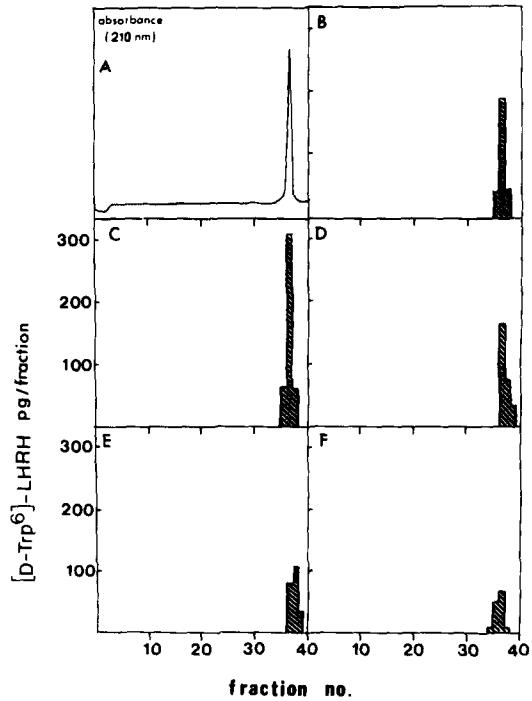

Fig. 3. HPLC behavior of standard and plasma extracted [D-Trp^6]-LHRH. (A) Standard [D-Trp^6]-LHRH dissolved in 11 mM TFA (50 μg/100 μl). (B) Standard [D-Trp^6]-LHRH added to plasma and Sep-Pak extracted. (C–F) Sep-Pak extracted dog plasma after intravenous injection, collected at various times: 20 min (C), 40 min (D), 75 min (E) and 150 min (F). [D-Trp^6]-LHRH was monitored by UV absorbance (A) or by RIA (B–F).

Kinetic studies

Intravenous injections. In both dogs, disappearance of immunoreactive [D-Trp^6]-LHRH displayed the same profile and could be fitted to a biexponential curve ($\log C = Ae^{-\alpha t} + Be^{-\beta t}$), the first and second terms corresponding respectively to the distribution and elimination of the peptide (Fig. 4). The slopes were calculated by the method of least square with half-lives ($t_{1/2}$ corresponding to $\ln \alpha/2$ (distribution) and $\ln \beta/2$ (elimination). Analysis of the curves yielded distribution $t_{1/2}$ of 12 min (dog A) and 10 min (dog B) and elimination $t_{1/2}$ of 84 min (dog A) and 119 min (dog B). Four samples collected at 20, 40, 75 and 150 min (dog A) were submitted to HPLC after Sep-Pak extraction. Fractionation of each extract showed a unique immunoreactive peak corresponding to [D-Trp^6]-LHRH (Fig. 3). Elimination half-life calculated from the 3 last samples after HPLC was 77 min. These results suggest that direct assay of plasma sample with antiserum 2050-11 reflected intact [D-Trp^6]-LHRH since elimination through Sep-Pak and HPLC purifications of any possible interference as proteins or cross-reacting substances, resulted in similar [D-Trp^6]-LHRH elimination rate.

Subcutaneous injections. Figure 5 shows [D-Trp^6]-LHRH concentrations after subcutaneous injection to dogs A2 to F2. After an absorption phase, [D-Trp^6]-LHRH plasma level reached a maximum between 45 and 60 min and declined exponentially until 12 h after injection. The resulting elimination half-life was 108 ± 9 min (mean ± S.E.M.; $n = 6$). Peptide concentration in the plasma was still consistent at 12 h and comparable to basal level at 24 h.

Long-acting formulations. In dogs A2 to F2, high [D-Trp^6]-LHRH level was reached 24 h after the injection of microcapsules, as shown in Fig. 6. This peak corresponded to rapid release of the peptide adsorbed on the outer part of the microcapsules. However between day 4 and day 31 we observed a plateau and the

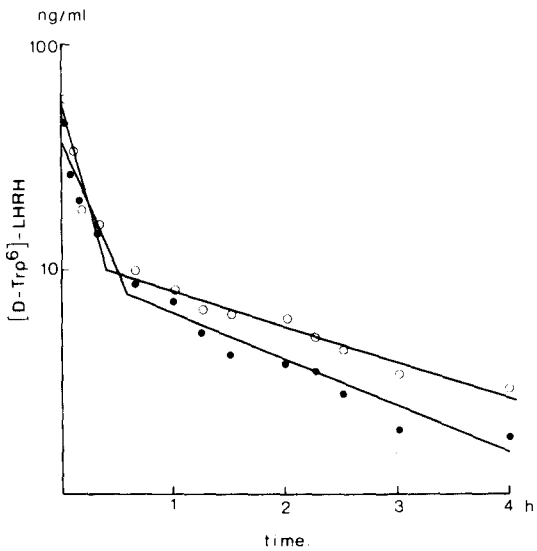

Fig. 4. [D-Trp^6]-LHRH plasma level after intravenous injection (100 μg) in dogs A1 (●) and A2 (○).

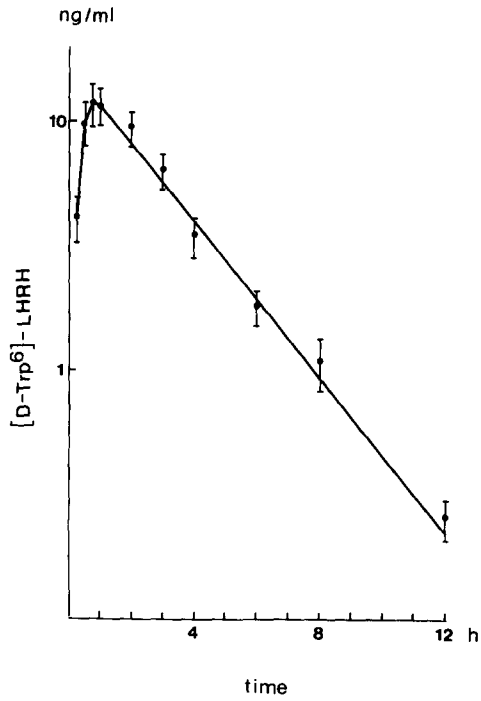

Fig. 5. [D-Trp^6]-LHRH plasma level after subcutaneous injection (100 μg) in 6 dogs (A2 to F2). Means ± S.E.M. (n = 6) are shown.

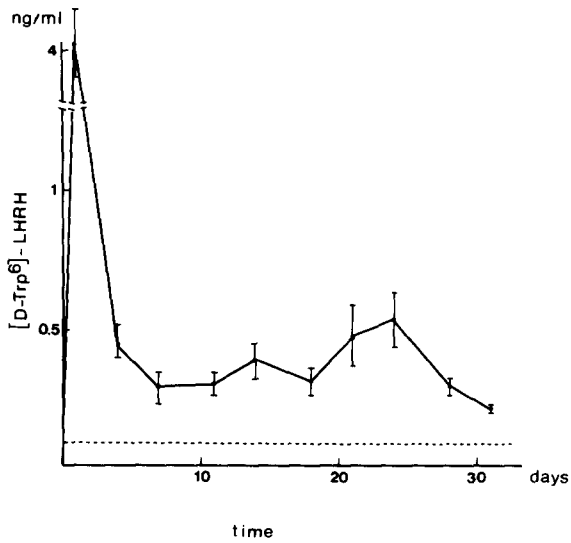

Fig. 6. [D-Trp^6]-LHRH plasma level after long-acting formulation (Cytotech batch 84-27) injection to the 6 dogs A2 to F2. Broken line represents basal level of [D-Trp^6]-LHRH-like immunoreactivity in dog plasma before injection. Means ± S.E.M. (n = 6) are shown.

TABLE II

Testosterone plasma level (ng/ml) in dogs A2 to F2 after long-acting formulation administration

Days after injection	A2	B2	C2	D2	E2	F2
Basal	0.76	1.78	1.81	1.60	0.63	1.48
1	1.43	4.16	3.79	3.90	4.58	4.42
4	<0.25	0.26	0.38	0.74	3.05	1.75
7	<0.25	<0.25	<0.25	<0.25	0.38	<0.25
11	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
14	<0.25	0.32	<0.25	<0.25	<0.25	<0.25
18	<0.25	0.42	<0.25	<0.25	<0.25	<0.25
24	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
28	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
31	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25

peptide concentration was similar to that observed between 8 and 10 h after the 100 μg subcutaneous injection. Stimulation of gonadotropin cells led to a rise in testosterone plasma concentration at 24 h but castration level was obtained between day 4 and day 7 and maintained until day 30 (Table II). In rats, [D-Trp^6]-LHRH concentrations displayed the same profile (Fig. 7). Although testosterone level in rat never reached the castrate level, there was, as in dogs, a stimulation at 24 h and a significant decrease over the assay period confirming an efficient [D-Trp^6]-LHRH release (Table III).

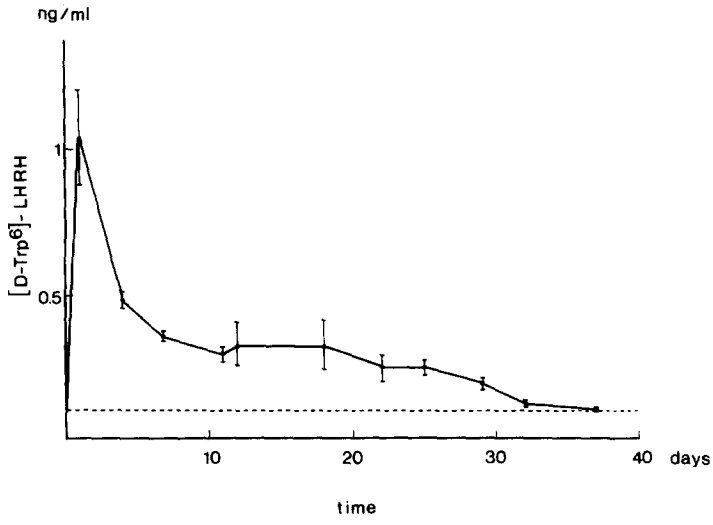

Fig. 7. [D-Trp^6]-LHRH plasma level after long-acting formulation (Cytotech batch 84-27) injection to 4 rats. Broken line represents basal level of [D-Trp^6]-LHRH-like immunoreactivity in rat plasma before injection. Means ± S.E.M. (n = 4) are shown.

TABLE III

Testosterone plasma level (ng/ml) in rats after long-acting formulation administration (n = 4) and in control rats (n = 8)

Days after injection	Treated animals	Controls
1	3.30 ± 1.31	0.75 ± 0.17
4	0.37 ± 0.08	1.36 ± 0.59
7	<0.25	1.16 ± 0.31
11	0.44 ± 0.12	1.20 ± 0.33
15	0.82 ± 0.41	1.14 ± 0.49
18	0.38 ± 0.04	1.29 ± 0.42
22	0.47 ± 0.10	1.20 ± 0.33
25	0.66 ± 0.13	1.68 ± 0.10
29	0.49 ± 0.10	0.93 ± 0.17
32	0.63 ± 0.09	1.35 ± 0.32
37	0.70 ± 0.08	0.82 ± 0.21

Values are the means ± S.E.M.

Discussion

We have developed a sensitive radioimmunoassay with two antisera against [D-Trp^6]-LHRH coupled to bovine serum albumin through glutaraldehyde or benzoquinone. These antisera present different characteristics. The first antiserum (2050-11) has a high sensitivity (detection limit of [D-Trp^6]-LHRH, 1 pg/tube) and the interferences with non specific material in unextracted plasma were negligible at dilutions used in the assays; however, the recognition of various LHRH fragments was appreciable.

The second antiserum (2069-12) recognized exclusively [D-Trp^6]-LHRH but its lower affinity contributes to high biological interferences which need to be eliminated through Sep-Pak purification. Since plasma sample extracts fractionated by HPLC contained a major (> 90%) immunoreactive peak corresponding to standard [D-Trp^6]-LHRH, the majority of measured material in direct plasma assay with the 2050-11 antiserum appeared to be the intact polypeptide form; accordingly, this antiserum was used in all direct assays. Disappearance of [D-Trp^6]-LHRH from dog plasma after intravenous or subcutaneous injection was found to be considerably slower than native LHRH [15]. Similar observations were made with other LHRH analogs [16–18].

In spite of the longer half-life, daily injections of [D-Trp^6]-LHRH do not allow the maintenance of a consistent blood level over 24 h. Using the release form, there is no gap during the period covered by the drug. Before clinical trials in human, we have tested the long-acting delivery system in dogs and rats. Although the dose injected per body weight was higher in rats than in dogs (300 and 180–240 μg/kg per month, respectively), the peptide level in rats was slightly lower than in dogs. This

difference may be explained by intraspecies variations. In both animals, the profile of testosterone concentration indicated the efficiency of the delivery system. In dogs, after long-acting formulation administration, plasma testosterone decreased quickly to castrate level. Compared to controls, rats only showed a 50% decrease, with low intraindividual variations. Recently, Schally et al. [19] have described a [D-Trp^6]-LHRH RIA and its application to a similar long-acting formulation. With a 750 μg/month dose per rat, they observed a higher and fluctuating [D-Trp^6]-LHRH level but the decrease in serum testosterone was more significant and remained 50 days after the administration. In humans with a 45 μg/kg per month dose, we have observed plasma levels similar to those obtained with 300 μg/kg per month in rats (unpublished data). In clinical trials, a dose of 3 mg in long-acting formulation induced a biological castration within 20 days which was maintained after monthly injections (unpublished data).

Before use in clinical trials, every batch of the long-acting formulation was tested in rats and displayed the same profile as batch 84-27 presented herein. Therefore, our radioimmunoassay, performed in rats to control the quality of long-acting forms of [D-Trp^6]-LHRH, allows us to predict the efficiency of the release in man and to assure a constant blood level of the peptide as a therapeutic agent.

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