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Radioimmunoassay for 6-D-tryptophan analog of luteinizing hormone-releasing hormone: Measurement of serum levels after administration of long-acting microcapsule formulations

(antiserum to [6-D-tryptophan]luteinizing hormone-releasing hormone/controlled release of peptide hormones)

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ABSTRACT **A sensitive and specific radioimmunoassay for [6-D-tryptophan]luteinizing hormone-releasing hormone ([D-Trp^6]LH-RH) was developed and used for following the rate of liberation of [D-Trp^6]LH-RH from a long-acting delivery system based on a microcapsule formulation. Rabbit antibodies were generated against [D-Trp^6]LH-RH conjugated to bovine serum albumin with glutaraldehyde. Crossreactivity with LH-RH was less than 1%; there was no significant cross-reactivity with other peptides. The minimal detectable dose of [D-Trp^6]LH-RH was 2 pg per tube. Intra- and interassay coefficients of variation were 8% and 10%, respectively. The radioimmunoassay was suitable for direct determination of [D-Trp^6]LH-RH in serum, permitting the study of blood levels of the analog after single injections into normal men and after once-a-month administration of microcapsules to rats. In men, 90 min after subcutaneous injection of 250 μg of the peptide, serum [D-Trp^6]LH-RH rose to 6–12 ng/ml. Luteinizing hormone was increased 90 min and 24 hr after the administration of the analog. Several batches of microcapsules were tested in rats and the rate of release of [D-Trp^6]LH-RH was followed. The improved batch of microcapsules of [D-Trp^6]LH-RH increased serum concentrations of the analog for 30 days or longer after intramuscular injection. This was accompanied by suppression of testosterone levels for more than 30 days. This radioimmunoassay should be of value for monitoring [D-Trp^6]LH-RH during long-term therapy.**

Superactive agonists of luteinizing hormone-releasing hormone (LH-RH) such as [D-Trp^6]LH-RH after chronic administration produce marked inhibitory effects through a process of "down regulation" of receptors and desensitization of the pituitary gonadotrophs (1–3). This inhibition of the pituitary–gonadal axis is manifested by a decrease in the secretion of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and gonadal steroids (1–3). These phenomena have been used for the treatment of prostate and mammary cancer, idiopathic precocious puberty, endometriosis, and other hormone-dependent disorders (4–10). The clinical efficacy of [D-Trp^6]LH-RH and related analogs for the treatment of prostate carcinoma has been well documented (4–9).

In these trials, superagonists of LH-RH were given daily by subcutaneous (s.c.) or intranasal routes (4–6). However, daily injections are inconvenient and intranasal administration is much less effective than parenteral (4–6). The use of [D-Trp^6]LH-RH for the treatment of hormone-dependent tumors and various disorders would be greatly enhanced by delivery systems capable of maintaining controlled levels of the peptide over an extended period of time (4). Recently, we have developed a long-acting delivery system based on a formulation of [D-Trp^6]LH-RH in microcapsules of poly(DL-lactide-co-glycolide) (11, 12). The injectable microcapsules were designed to release 100 μg of [D-Trp^6]LH-RH per day over a 30-day period (11, 12). This approach is convenient and efficacious. To monitor the release of [D-Trp^6]LH-RH from microcapsules, we developed a sensitive and specific RIA for [D-Trp^6]LH-RH in unextracted serum. This RIA is described in this paper, and studies on pharmacokinetics of liberation of [D-Trp^6]LH-RH from the microcapsules are also reported.

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MATERIALS AND METHODS

Analog and Microcapsules. [D-Trp^6]LH-RH was synthesized by solid-phase methods and supplied by Debiopharm (Lausanne, Switzerland). Microcapsules of [D-Trp^6]LH-RH were prepared by a phase-separation process (11, 12). Prototype batch C 109-104 was from Southern Research Institute (Birmingham, AL). Batch 7 was from Cytotech (Martigny, Switzerland). The microcapsules were spherical particles consisting of [D-Trp^6]LH-RH (1.8–3.6%, wt/wt) dispersed throughout a polymeric matrix of poly(DL-lactide-co-glycolide) (96.4–98.2%, wt/wt) (11).

Immunogen Preparation. A modification of a coupling procedure described elsewhere (13) was used. Four hundred micrograms of [D-Trp^6]LH-RH in 0.4 ml 0.01 M acetic acid was mixed with 0.6 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 2 mg of bovine serum albumin (Sigma, A-7888), and 130 μl of 0.021 M glutaraldehyde (Sigma, G-5882) was added with constant stirring. After 4 hr of stirring, 2 ml of 0.1 M phosphate buffer, pH 7.0, and 3 ml of Freund's complete adjuvant (GIBCO, no. 660-5721) were added. The entire mixture was emulsified.

Immunization. Two female New Zealand White rabbits were injected with freshly conjugated and emulsified bovine serum albumin-peptide/Freund's adjuvant mixture. The inoculum (equivalent to 200 μg of [D-Trp^6]LH-RH per rabbit) was injected intramuscularly, intraperitoneally, and subcutaneously. Booster preparations composed of incomplete Freund's adjuvant (GIBCO, no. 660-5720) emulsified with 100 μg of [D-Trp^6]LH-RH and 1 mg of bovine serum albumin were given after 3 weeks, and thereafter monthly. The animals were bled prior to initiation of immunization and 2–3 weeks after each booster.

Radioiodination. [D-Trp^6]LH-RH was iodinated by a modi-

Abbreviations: LH, luteinizing hormone; LH-RH, luteinizing hormone-releasing hormone; B_0, percent of total added radiolabeled ligand bound to antibody in the absence of unlabeled ligand; ED_{20}, ED_{50}, and ED_{80}, the effective dose estimated at 20%, 50%, and 80% of the B_0 value; IU, international unit.

fication of the the chloramine-T method (14): To 5 μg of [D-Trp^6]LH-RH in 5 μl of 0.1 M acetic acid were added sequentially 40 μl of 0.5 M sodium phosphate buffer at pH 7.5, 1 mCi (37 MBq) of Na^{125}I, and 10 μg of chloramine-T in 10 μl. After 30 sec, the reaction was terminated by adding 100 μl of 25% (wt/vol) solution of human serum albumin.

Purification of Labeled [D-Trp^6]LH-RH. Labeled hormone was separated from free iodine by gel filtration on a 1 × 20 cm column of Sephadex G-25, using 0.1 M acetic acid containing 0.25% bovine serum albumin as eluant. One-milliliter fractions were collected.

RIA Procedure. Polypropylene tubes (Scientific Products, 12 × 75 mm, no. T1226-12) were used throughout to prevent adsorption of the peptide. The assay buffer consisted of 0.025 M EDTA, 0.01 M sodium phosphate at pH 7.6, 0.14 M NaCl, and 0.35% human serum albumin.

Standards. Aliquots of [D-Trp^6]LH-RH solution, 1 μg/5 μl of 0.1 M acetic acid, were pipetted into the tubes, stored at −70°C, and diluted with buffer before each assay. The range of the standard curve was from 4.1 to 2500 pg/100 μl.

Assay procedure. The incubation mixture consisted of 100 μl of standard and 400 μl of assay buffer or 20 μl of serum sample and 480 μl of assay buffer, 25,000 dpm of ^{125}I-labeled [D-Trp^6]LH-RH in 100 μl, and 100 μl of antiserum to [D-Trp^6]LH-RH (SV-112, at a working dilution of 1:5000). The tubes were incubated at 4°C for 24 hr. Bound and free fractions were separated by a polyethylene glycol-facilitated double antibody method using 500 μl of 10% (wt/vol) polyethylene glycol (M_r = 3350) and 100 μl of 5% goat antiserum to rabbit gamma globulin (Antibodies Inc., no. 1156) per tube. After centrifugation at 2900 × *g* for 20 min, the supernatants were decanted and the radioactivities of the precipitates were measured in a 10-detector γ counter (Micromedic Systems, Horsham, PA). All samples were assayed in duplicate.

RIA Validation. Assay sensitivity, specificity, accuracy, and precision as well as the effects of the presence of serum in the assay system were evaluated.

Sensitivity. Twenty replicates of the zero standard were assayed in one assay run.

Specificity. Various analogs and fragments of LH-RH were assayed at concentrations varying by four orders of magnitude. Several other peptide hormones were also tested.

Accuracy. Fractional recovery studies were performed with rat and human serum. [D-Trp^6]LH-RH was added to a pool of serum at four dose levels and the mixture was assayed. Dilutional parallelism was evaluated by making serial dilutions of a serum pool containing [D-Trp^6]LH-RH at approximately 15 ng/ml, using peptide-free serum as the diluent.

Recovery of [D-Trp^6]LH-RH and dilutional parallelism were also studied with methanol extracts of the serum, using a 10:1 (vol/vol) ratio of methanol to serum. In another experiment, [D-Trp^6]LH-RH concentrated from serum by using Sep-Pak cartridges (Waters Associates) was also tested, using 50% acetonitrile/0.05% trifluoroacetic acid (vol/vol) as the elution solvent.

Precision. Twenty replicates of a pool of normal rat serum and 20 replicates of a pool of rat serum containing [D-Trp^6]LH-RH were assayed in 1 run to evaluate intra-assay precision and in 10 assay runs to determine inter-assay precision.

Effects of serum. Different aliquots of peptide-free serum were added to the standard curve to test the effect of increasing serum volume on the assay system.

Determination of Effects of Acute Administration of [D-Trp^6]LH-RH in Men. Five healthy adult men volunteered for this study and gave informed consent. [D-Trp^6]LH-RH was given s.c. at a dose of 250 μg. Blood samples were taken from the antecubital vein before (basal values) and 90 min and 24 hr after administration. The serum was stored frozen at −20°C. Human LH was measured with a commercially available RIA kit (Radioassay Systems Laboratories, Carson, CA).

Experiments in Rats. Adult male Sprague–Dawley rats received single intramuscular injections of various batches of microcapsules of [D-Trp^6]LH-RH (experiment 1, batch C 109-104, Southern Research Institute; experiment 2, batch 7, Cytotech). The microcapsules, in 28- to 40-mg portions calculated to release about 25 μg per day for 30 days, were suspended in disposable syringes in 0.7 ml of injection vehicle consisting of 2% CM-cellulose and 1% Tween 20 in water (11, 12). The suspension was mixed thoroughly on a Vortex mixer and injected through an 18-gauge needle into the thigh muscle. Control rats were injected with the vehicle. Six to 12 rats were used per group.

Blood samples of about 0.7 ml were taken from the jugular vein daily for the first 7 days and periodically thereafter for 20–40 days after the injection. The serum was collected and stored frozen at −20°C until assayed for [D-Trp^6]LH-RH. Serum samples were also assayed for rat LH (using the RIA kit provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases) and for testosterone (using a kit from Radioassay Systems Laboratories).

Statistical evaluations were made with Duncan's new multiple-range test (15).

RESULTS

Antiserum. Of the two rabbits immunized, only one produced a useful antiserum. The second bleeding of this animal yielded an antiserum (SV-112) with a sufficiently high titer (1:35,000, final dilution) and affinity constant (K_a = 2.4 × 10^{11} M^{-1}) to be used in an RIA. This dilution produced a B_0 value of approximately 30%.

Radioiodination. Since the specific activity of the [D-Trp^6]LH-RH labeled by the chloramine-T method was almost twice as high as that of the analog iodinated with lactoperoxidase (1426 μCi/μg vs 780 μCi/μg, respectively), the chloramine-T method was used.

Purification of Labeled [D-Trp^6]LH-RH. The central portion of the peak of the ^{125}I-labeled [D-Trp^6]LH-RH eluted from the Sephadex G-25 column (V_e/V_0 = 5.5) was pooled and stored at 4°C. This fraction possessed the highest degree of immunoreactivity. The material was repurified by the same method before each assay. No loss of immunoactivity was observed for up to 3 weeks after iodination.

Radioimmunoassay Characteristics. A typical assay produced a standard curve (Fig. 1) with these characteristics: nonspecific binding, 3.1%; B_0, 32.4%; slope, −0.80934;

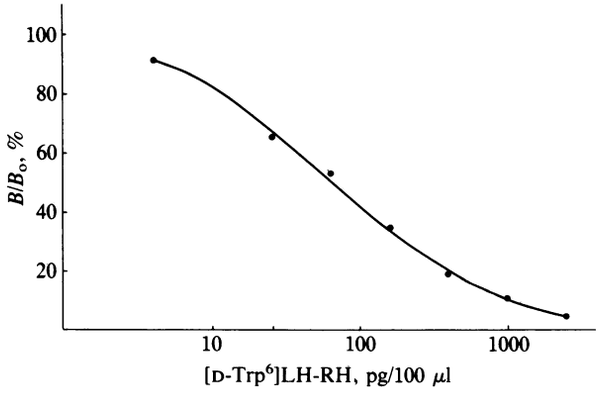

FIG. 1. Representative standard curve of the [D-Trp^6]LH-RH RIA.

Table 1. Crossreactivity of LH-RH fragments and analogs and other peptides with [D-Trp^6]LH-RH antiserum SV-112

Peptide	Crossreactivity, %
LH-RH	0.8
H-Ser-Tyr-Gly-Leu-OH	0.16
H-Leu-Arg-Pro-Gly-NH_2	0.08
[D-Trp^6]LH-RH ethyl amide	0.05
D-Arg antagonist Ac-[D-*p*-Cl-Phe^{1,2}, D-Trp^3, D-Arg^6, D-Ala^{10}]LH-RH	0.02
<Glu-His-Trp-OH, H-Trp-Ser-Tyr-Gly-Leu-OH, [D-Ser(Bu^t)^6]LH-RH ethyl amide, <Glu-His-Trp-Ser-Tyr-Gly-Leu-OH, [D-Leu^6]LH-RH ethyl amide, [L-5F-Trp^8, D-Cys^{14}]somatostatin, corticotropin, somatostatin-14, somatostatin-28, glucagon, gastrin, motilin, insulin, vasoactive intestinal peptide, gastric inhibitory peptide, secretin, growth hormone-releasing hormone, and corticotropin-releasing factor	<0.005

ED_{20}, 406 pg; ED_{50}, 71 pg; ED_{80}, 12 pg. The interassay coefficient of variation for the standard curve was 4.9% (n = 20).

Radioimmunoassay Validation. *Sensitivity.* The minimal detectable dose was found to be 2 pg per tube, based on the dose estimated by the 95% confidence limits for the cpm of the zero standard (n = 20).

Specificity. The specificity of the antiserum is given in Table 1. LH-RH showed a crossreactivity of 0.8%. LH-RH tetrapeptide fragments Leu-Arg-Pro-Gly-NH_2 and Ser-Tyr-Gly-Leu-OH crossreacted 0.08% and 0.16%, respectively, but other fragments of LH-RH reacted less than 0.005%. An LH-RH antagonist crossreacted 0.02% and [D-Trp^6]LH-RH ethyl amide, only 0.05%. Other LH-RH agonists and various peptides crossreacted less than 0.005%.

Accuracy. The results of the fractional recovery study are presented in Table 2. The recoveries of [D-Trp^6]LH-RH were 74–106% for unextracted serum and 67–75% for the methanol extracts. Recovery of [D-Trp^6]LH-RH added to serum and concentrated by the Sep-Pak system was less than 30%. The results of the dilutional parallelism study are presented in Fig. 2. Good parallelism was demonstrated in extracted and unextracted serum.

Table 2. Recovery of [D-Trp^6]LH-RH added to rat serum or human serum and assayed directly and after methanol extraction

Serum	Methanol extraction	[D-Trp^6]LH-RH, pg Added	[D-Trp^6]LH-RH, pg Recovered	Recovery, %
Rat	−	50	34	68
		100	70	70
		500	409	82
		1000	767	77
				Mean 74 ± 3.2
Rat	+	50	28	56
		100	52	52
		500	341	68
		1000	931	93
				Mean 67 ± 9.2
Human	−	50	55	110
		100	76	76
		500	552	110
		1000	1289	129
				Mean 106 ± 11.0
Human	+	50	29	58
		100	61	61
		500	384	77
		1000	1044	104
				Mean 75 ± 10.5

Values for extracted sera were corrected for losses. Means are presented ±SEM.

Precision. Intra- and interassay coefficients of variation for a pool of normal rat serum (mean = 3.8 pg/20 μl) were 16% and 18%, respectively. Intra- and interassay coefficients of variation for a pool of rat serum with increased [D-Trp^6]LH-RH levels (mean = 500 pg/10 μl) were 8% and 10%.

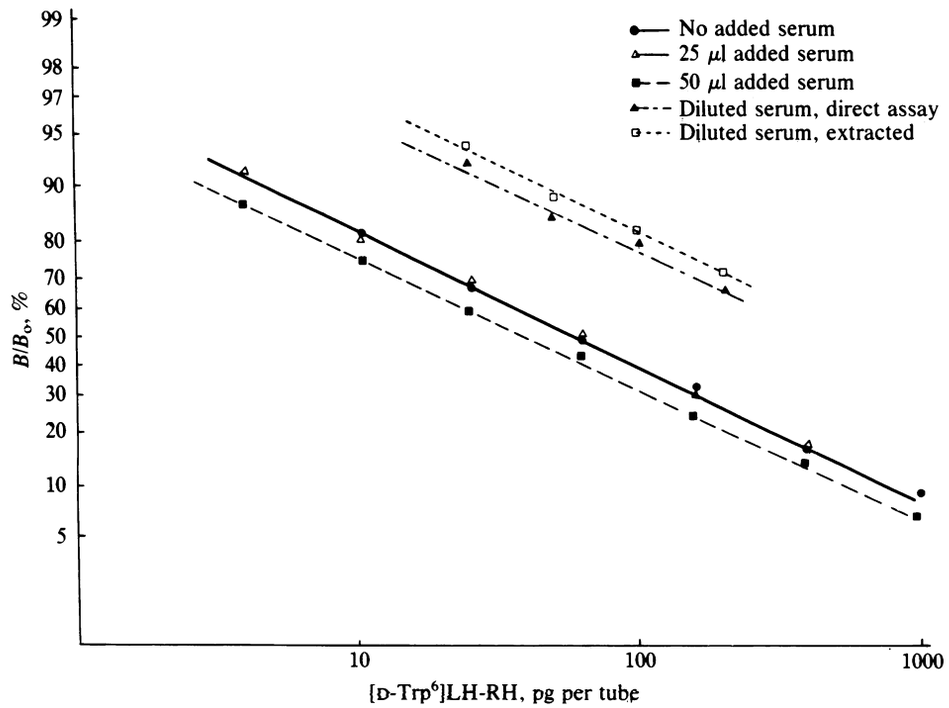

FIG. 2. Dilutional parallelism of [D-Trp^6]LH-RH in unextracted and extracted rat serum, and effect of adding increasing amounts of serum to the standard RIA.

Table 3. Serum concentrations of [D-Trp^6]LH-RH and LH in men after administration of 250 μg of [D-Trp^6]LH-RH

Subject	[D-Trp^6]LH-RH, ng/ml			LH, mIU/ml		
	Basal	90 min	24 hr	Basal	90 min	24 hr
1	0	6.42	0.09	12.6	30.5	22.3
2	0.11	9.25	0.68	12.8	28.7	25.1
3	0.56	12.10	0.37	12.3	72.4	50.7
4	0.12	9.74	0.61	8.5	50.5	56.3
5	0.16	8.60	0.36	12.6	49.8	51.5
Mean	0.2	9.2	0.42	11.8	46.4	41.2
SEM	±0.1	±0.9	±0.1	±0.8	±7.9	±7.2
P		<0.01			<0.01	<0.01

IU, international unit.

Effects of serum. There was little difference in the standard curve when 10 μl or 25 μl of serum was added to the standard concentrations of [D-Trp^6]LH-RH in the assay tubes. However, after the addition of 50 μl of serum, there was a displacement of the standard curve to the left (Fig. 2).

[D-Trp^6]LH-RH Levels in Men After s.c. Injections. The results of the experiment on measurement of [D-Trp^6]LH-RH in blood of five normal men are shown in Table 3. Ninety minutes after s.c. injection of 250 μg of [D-Trp^6]LH-RH, the serum concentration of this peptide was 9.2 ± 0.9 ng/ml ($P < 0.01$ vs. basal) and 24 hr after injection, 0.4 ± 0.1 ng/ml. The basal serum levels were below the detection limits of the assay (0.2 ± 0.1 ng/ml). The basal serum LH concentration was 11.8 ± 0.8 mIU/ml. LH rose to 46.4 ± 7.9 mIU/ml 90 min after the administration of analog, and 24 hr after administration the LH levels were still elevated (41.2 ± 7.2 mIU/ml). The 90-min and 24-hr values were significantly higher ($P < 0.01$) than the basal values.

[D-Trp^6]LH-RH Levels After Administration of Long-Acting Microcapsules to Rats. Various experiments were carried out with several batches of microcapsules, two of which are described here. Administration of an early prototype of microcapsules (Southern Research Institute lot C 109-104) resulted in a biphasic increase of [D-Trp^6]LH-RH levels in rats (Fig. 3). Within 24 hr, serum [D-Trp^6]LH-RH reached its peak, and it steadily decreased to its nadir on day 5. The second phase of release began on day 6, and an increase of [D-Trp^6]LH-RH could be seen up to day 14. The values for days 15–32 are not available, but by days 33–36 the amounts fell below the detection limits of the assay.

The improved batch of microcapsules (Cytotech lot 7) produced a protracted elevation of serum [D-Trp^6]LH-RH over a period of at least 30 days (Fig. 4). The profile of this release was biphasic and different from that observed for batch 1 (Fig. 3). The peak of phase 1 release was on day 5 and that of phase 2 was on day 18. High initial release seen with the early batches of microcapsules on days 1–5 after the injection did not occur in the case of the improved microcapsules.

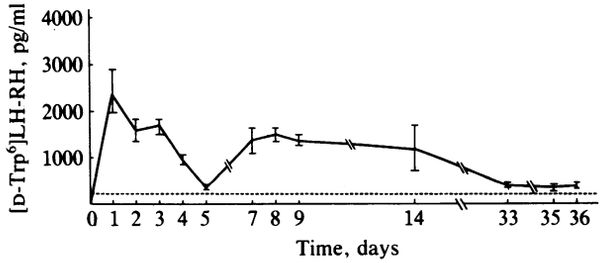

FIG. 3. [D-Trp^6]LH-RH release from a prototype batch of microcapsules. Broken line represents lower limit of detectability. Bars represent SEM.

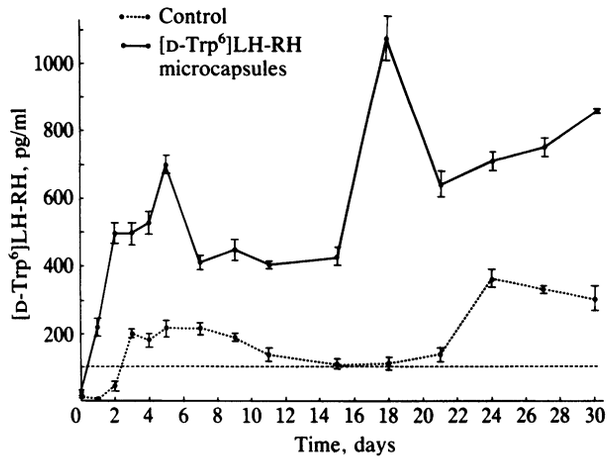

FIG. 4. [D-Trp^6]LH-RH release from improved batch of microcapsules. Broken line represents lower limit of detectability. Bars represent SEM; $n = 10$ for both groups. Note expanded ordinate compared with Fig. 3.

The release of the [D-Trp^6]LH-RH from these microcapsules was more evenly distributed over a period of about 30 days. The high background levels of [D-Trp^6]LH-RH observed in the control animals in this experiment (Fig. 4) represent measurements around the detection limits of the assay and may be due to nonspecific effects of those sera. Serum LH values in rats injected with microcapsules were highest on day 1 (Table 4) and declined to basal by day 5. Serum testosterone (Table 4) was elevated on day 1 but fell to levels below normal by day 3 and remained suppressed for 30–40 days. This suppression of serum testosterone demonstrates that the microcapsules exerted biological effects for at least 30 days. The depression of serum testosterone in the presence of sustained LH levels has been observed previously and suggests that chronic administration of LH-RH agonist suppresses the gonads more than the pituitary or that the immunologically active LH detected by RIA may have reduced biological activity (16).

DISCUSSION

The development of long-acting delivery systems for peptide hormones must be accompanied by sensitive and specific techniques for monitoring of serum levels. The RIA developed by us for [D-Trp^6]LH-RH appears to fulfill the criteria for such a technique.

[D-Trp^6]LH-RH is probably bound to macromolecular components of serum, as is LH-RH (17). This usually requires an extraction step before RIA can be performed. However, the combination of a specific antiserum with high affinity and a labeled peptide with considerable specific activity permits the RIA of [D-Trp^6]LH-RH in unextracted serum. Previously, an RIA for [D-Trp^6]LH-RH was described in which 100 μl of unextracted serum was used; however, the contribution of serum proteins to experimental error was not assessed (18). Although in our method 50 μl or more of serum will cause a shift in the standard curve, the RIA is sensitive enough to monitor the analog in only 20 μl of serum. That low levels of [D-Trp^6]LH-RH, which approach the limit of sensitivity of the assay, were correctly detected by such direct assay was confirmed by the continued suppression of serum testosterone in rats. Apparently, even these low serum levels of [D-Trp^6]LH-RH can exert the desired biological effects.

The results of a direct RIA on serum were superior to the assay of methanol extracts. This might be important in monitoring [D-Trp^6]LH-RH in large clinical trials, because an ex-

Table 4. Serum LH and testosterone in rats treated with [D-Trp^6]LH-RH microcapsules

Day	LH, ng/ml	Testosterone, ng/ml
0	0.62 ± 0.11	0.96 ± 0.24
1	2.33 ± 0.16	2.67 ± 0.17
2	1.43 ± 0.11	0.88 ± 0.12
3	1.18 ± 0.12	0.26 ± 0.06
4	0.93 ± 0.12	0.17 ± 0.04
5	0.69 ± 0.13	0.18 ± 0.06
7	0.69 ± 0.05	0.15 ± 0.05
9	0.66 ± 0.04	0.21 ± 0.07
11	0.71 ± 0.10	0.10 ± 0.02
13	0.72 ± 0.16	0.20 ± 0.05
15	0.59 ± 0.04	0.12 ± 0.04
17	0.68 ± 0.14	0.18 ± 0.06
21	0.67 ± 0.06	0.16 ± 0.04
24	0.76 ± 0.08	0.32 ± 0.09
27	0.72 ± 0.11	0.21 ± 0.05
30	—	0.22 ± 0.05
36	—	0.30 ± 0.05
41	—	0.21 ± 0.02
46	—	0.33 ± 0.13
50	—	0.48 ± 0.10
56	—	0.57 ± 0.09
60	—	0.40 ± 0.09

Microcapsules were Cytotech lot 7. LH values are expressed in terms of rat LH RP-2 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. All values are mean ± SEM for five animals.

traction step would impose a heavy workload. Potential clinical application of this method is shown by satisfactory recoveries of [D-Trp^6]LH-RH added to human serum and by the successful use of the RIA for the measuring of [D-Trp^6]LH-RH levels in men after s.c. injection.

Preliminary observations indicate that this RIA could be used after ethanol extraction for measuring blood levels of [D-Trp^6]LH-RH in patients with prostate cancer receiving daily s.c. injections or monthly administration of microcapsules.‡

Our results also indicate that the improved batches of microcapsules release [D-Trp^6]LH-RH in a continuous controlled fashion over a 30-day period. Clinical observations in men with prostate cancer and women with endometriosis indicate that the plasma levels of [D-Trp^6]LH-RH after an intramuscular administration of microcapsules designed to release about 100 μg/day for 30 days are high enough to exert specified therapeutic effects.‡

The advantage of using LH-RH agonists for treatment of prostate cancer and other hormone-dependent diseases is a relative lack of side effects (4–9). The development of injectable microcapsules for intramuscular administration at monthly intervals should make the treatment with [D-Trp^6]LH-RH more convenient and possibly more efficacious as compared to discontinuous administration and should also better ensure patient compliance (4, 7, 11).

‡Roger, M., Duchier, J., Lahlou, N., Mauvernay, R. Y. & Schally, A. V., Seventh International Congress of Endocrinology, July 1–7, 1984, Quebec City, PQ, Canada, p. 1288 (abstr. no. 2055).

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