Long-Term Stability of Aqueous Solutions of Luteinizing Hormone-Releasing Hormone Assessed by an *In Vitro* Bioassay and Liquid Chromatography

YI-FAN SHI *, RICHARD J. SHERINS *, DAVID BRIGHTWELL *, JOSEPH F. GALLELLI ‡, and DULAL C. CHATTERJI ‡§x

Received December 13, 1982, from the *Developmental Endocrinology Branch, National Institute of Child Health and Human Development, and the ‡Pharmaceutical Development Service Section, Pharmacy Department, The Clinical Center, National Institutes of Health, Bethesda, MD 20205. Accepted for publication May 20, 1983. §Present address: Research and Development, Lypho-Med Inc., Melrose Park, IL 60160.

Abstract □ The stability of aqueous solutions of luteinizing hormone-releasing hormone (LHRH) after extended storage at various temperatures was investigated using a newly developed HPLC assay and an *in vitro* dispersed pituitary cell culture bioassay. Good correlations were obtained between the potency obtained by HPLC and bioassay in samples stored at 37°C or subjected to different stress conditions. No significant decrease in activity of LHRH was observed in aqueous solutions stored at 37°C for up to 10 weeks, at 4°C for 2 years, or subjected to repeated freezing and thawing for 5 d. Heating to 60°C in sterile pH 9.0 buffer up to 11 d and storage at ambient temperature in nonsterile solution for 4 months produced well-distinguished degradation products and a decrease in potency. It is concluded that sterile aqueous solutions of LHRH are stable for at least 10 weeks at 37°C and, thus, could be reliably used for chronic administration when long-term stability at body temperature is important.

Keyphrases □ Luteinizing hormone-releasing hormone—stability, aqueous solutions, *in vitro* bioassay, HPLC □ Stability—LHRH, aqueous solutions, *in vitro* bioassay, HPLC □ Degradation—LHRH aqueous solutions, *in vitro* bioassay, HPLC

Luteinizing hormone-releasing hormone (LHRH), isolated from porcine hypothalami by Schally *et al.* (1), was found to release both luteinizing hormone and follicle-stimulating hormone from the pituitary of several species, including humans (1, 2). LHRH has recently been found to be clinically useful in stimulating gonadotropin release, testicular development (3–5), and follicular maturation and ovulation (6, 7) in hypogonadotropia in humans, especially when small doses of LHRH are administered chronically by intermittent intravenous injection (8–10) or by a portable infusion pump (11–13).

We have evaluated the stability of LHRH solutions under commonly used storage conditions and after long-term exposure to body temperature (37°C) to determine how long and under what conditions LHRH solutions might be utilized for chronic hormone administration. LHRH activity was assayed by *in vitro* pituicyte bioassay as well as a newly developed HPLC technique.

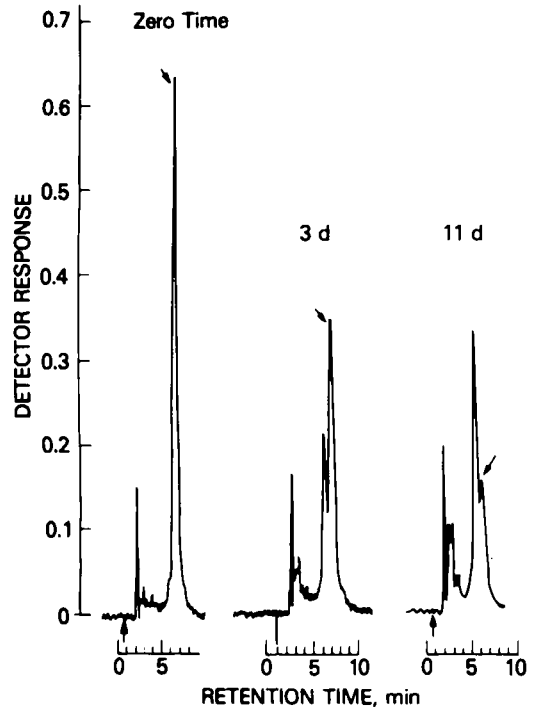

Figure 1—*HPLC profile of LHRH solutions prepared in 0.01 M borate buffer (pH 9.0) kept at 60°C for 0, 3, and 11 d. The arrows indicate intact LHRH peaks.*

EXPERIMENTAL

General Experimental Protocol—Sterile stock solutions of synthetic LHRH[1] were prepared at a concentration of 1 mg/mL in distilled water or 0.01 M borate buffer (pH 9.0). Aliquots (1 mL) of the stock solution were dispensed into 2-mL glass vials and sealed with polytef-lined stoppers. The samples were stored at various temperatures as specified below. At selected time intervals, two individual vials were removed and stored at −20°C for

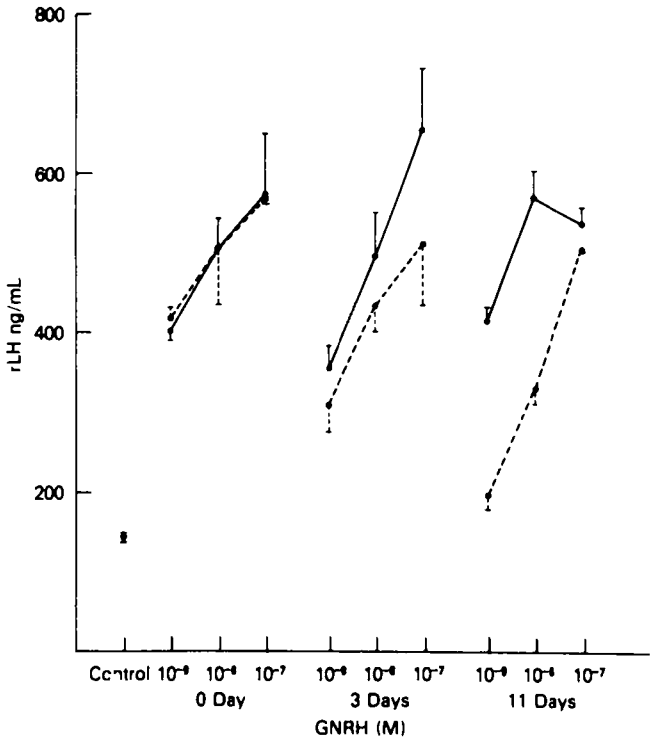

Figure 2—*Luteinizing hormone release from dispersed rat pituitary cells in culture following incubation with LHRH solutions kept at 60°C for 0, 3, and 11 d. Key: (—) in distilled water; (- - -) in buffer (pH 9.0).*

[1] Synthesized by Dr. Erhard Gross at NIH; lot CCD 332 78-135.

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Table I—Stability of LHRH at 60°C

	Day	Potency, % HPLC	Potency, % Bioassay	
	0	100	100	
Distilled water	3	96	118	($p > 0.25$)
	11	96	142	($p > 0.25$)
Buffer pH 9.0	3	40	15	($p < 0.02$)
	11	5	7	($p < 0.01$)

subsequent analysis: one was analyzed by HPLC and the other by an *in vitro* bioassay using dispersed rat anterior pituitary cells in culture. The potency of all treated samples was compared with freshly prepared LHRH solutions.

Experiment 1—To check the specificity of the HPLC analysis, stock solutions of LHRH in distilled water and in borate buffer pH 9.0 were heated to 60 ± 2°C in a constant-temperature oven for 0, 3, and 11 d. The potency of the samples was anlayzed by both HPLC and bioassay.

Experiment 2—To provide a different set of degradation products to test the specificity of HPLC assay, a degraded LHRH solution was prepared by leaving a nonsterile solution of LHRH at ambient temperature for 4 months. Three mixtures of LHRH solutions were then prepared: (A) 90% fresh LHRH + 10% degraded LHRH, (B) 90% fresh LHRH + 10% normal saline, and (C) 100% degraded LHRH solution. Solution B was used as the control sample.

Experiment 3—To determine the long-term stability at body temperature, stock LHRH solutions in distilled water were kept at 37 ± 0.1°C in a constant-temperature water bath, and the activity of LHRH was assayed by HPLC and pituitary cell culture. Samples were incubated for 0, 4, 6, and 10 weeks in the first experiment and for 0, 10, and 27 weeks in a second study.

Experiment 4—To obtain the long-term shelf life, sterile solutions of LHRH were stored for 2 years at 4°C. The LHRH potency was determined by the HPLC method alone.

Experiment 5—To evaluate the effect of accidental freezing and thawing, one set of samples was stored at −20°C in a freezer but subjected to a 1-h thaw at room temperature every day for 5 d. The potency of the frozen and thawed samples was compared with the original control vials, which remained frozen until HPLC assay.

Liquid Chromatographic Assay of the LHRH Solution— HPLC was performed on a 25 cm × 4-6 mm i.d. reverse-phase column[2] using a liquid chromatograph[3] equipped with a variable-wavelength detector[4] and a fixed-volume injector[5]. A mobile phase of acetonitrile–acetic acid–sodium hexylsulfonate–water (35:0.1:0.2:64.7) at a flow rate of 1.6 mL/min was used as the eluant. Retention time of LHRH was 6.0 min under these conditions.

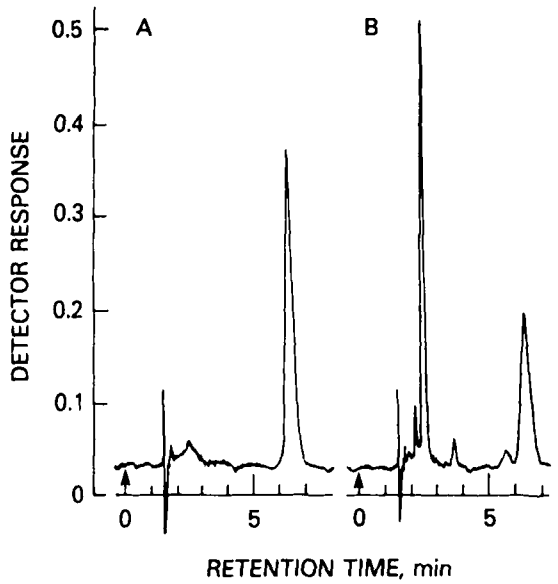

Figure 3—*HPLC profile of nonsterile LHRH solutions stored at ambient temperature. Key: (A) time zero; (B) 4 months.*

[2] Zorbax-C8; DuPont Instruments, Wilmington, Del.
[3] Model 3500 B; Spectra-Physics, Santa Clara, Calif.
[4] S.F. 770; Schoeffel Instruments, Western, N.J.
[5] Velco, Houston, Tex.

Table II—Specificity of the HPLC Assay

Solution	Potency, % HPLC	Potency, % Bioassay	
A	106	106	($p > 0.25$)
B	100	100	($p < 0.001$)
C	55	31	

Testosterone, which was used as internal standard in a few runs to check the quantitation accuracy, had a retention time of 11.5 min. The wavelength for quantitation was 220 nm.

Solutions of LHRH were diluted quantitatively to 20 μg/mL, and 50 μL of this solution was injected using the fixed-loop injector. Testosterone, when added to check the accuracy, was added at a concentration of 10 μg/mL in the mobile phase used for dilution of stock LHRH solutions. Either the peak height of LHRH or peak height ratio of LHRH to testosterone was used for quantitation. The potency of the treated samples was compared with the freshly prepared samples.

Bioassay of the LHRH Solutions—The bioactivity of LHRH was assessed by quantitating the release of luteinizing hormone into the media of dispersed rat anterior pituitary cells placed into short-term monolayer culture according to the method of Vale *et al.* (14). The control and experimental LHRH samples were added to the cell culture in the dose range of 10^{-7}–10^{-10} M for 3 h, and the medium was then aspirated, centrifuged to remove any suspended cells, and the luteinizing hormone concentration was measured by double-antibody RIA using LHRH-1 as standard (15). Reagents were provided by the NIAMDDK National Hormone and Pituitary Program. All samples from one experiment were measured individually in duplicate in a single assay to eliminate between-assay variation. The assay detection limit was 2 ng/mL.

Statistical Analysis—All bioassay results are presented as the mean ± *SEM*. Potency estimates were determined within each cell culture experiment relative to the LHRH stock solution prepared on day 0. Each LHRH dose-response curve was first tested for linearity (*t* test) and then for parallelism (*F* test) to the control curve prior to the estimation of potency. The relative precision (x) for symmetrical experimental designs was determined according to the method of Finney (16).

RESULTS AND DISCUSSION

Specificity of the Liquid Chromatographic Method and Its Correlation with the Bioassay—The HPLC method gave sharp and reproducible peaks for LHRH, and the height of the LHRH peak was directly proportional to the concentration of LHRH in a series of freshly prepared solutions. The HPLC profile of LHRH in borate buffer pH 9.0 heated to 60°C showed a degradation product peak which was well separated from the intact LHRH peak (arrow, Fig. 1). The small shoulder (corresponding to the retention time of the degradation product) in the time-zero sample in Fig. 1 is, however, attributible to a chromatographic artifact. Purity of the LHRH peak was verified by comparison of the retention times and peak height ratios at 210, 220, and 230 nm of the sample peaks to those from a freshly prepared solution of LHRH. Solutions of LHRH in distilled water were stable after being heated to 60°C for up to 11 d. By contrast, significant loss of integrity was observed at pH 9.0 during the same time interval. The results of the HPLC assay correlated well with those obtained by bioassay (Fig. 2, Table I). The potency of the mild

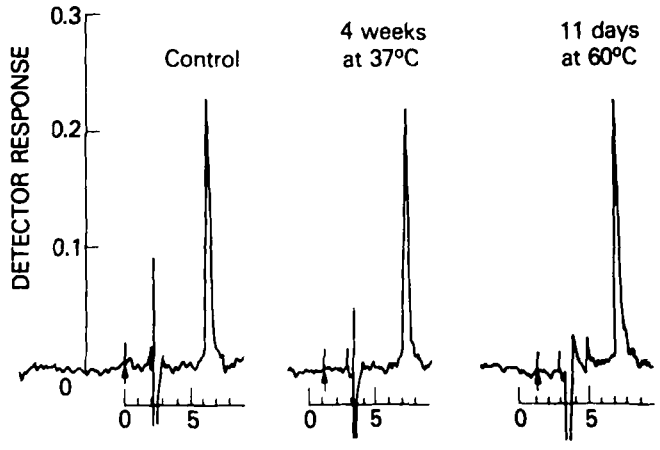

Figure 4—*HPLC profile of LHRH solutions prepared in distilled water kept at 60°C for 11 d or at 37°C for 4 weeks.*

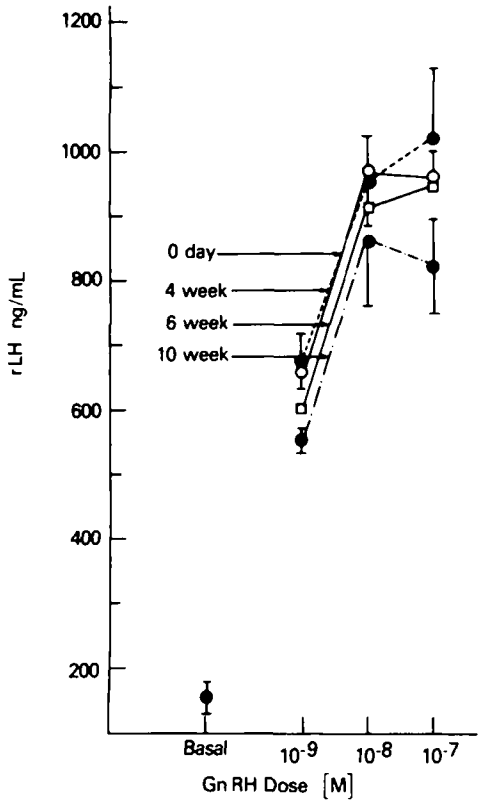

Figure 5—*Luteinizing hormone release from dispersed rat pituitary cells in culture following incubation with LHRH solutions prepared in distilled water (pH 6) and kept at 37°C for 0, 4, 6, and 10 weeks.*

alkaline solution of LHRH stored at 60°C for 11 d was 5% by the HPLC assay and 7% by bioassay.

The HPLC profile of the degradation product of the nonsterile LHRH solution stored at ambient temperature for 4 months was different from that obtained from LHRH solution stored at 60°C in borate buffer (Fig. 3). The potencies of this degraded LHRH product were 55 and 31% when assayed by HPLC and pituitary cell culture, respectively (Table II). There was no significant difference in the potency between LHRH solutions mixed with normal saline or the degradation product at a final concentration of 10% (Table II).

Analysis of the HPLC assay shows that under the conditions tested in this study (*a*) it gives reproducible LHRH peaks both in terms of retention time and peak height ratio at three different wavelengths, (*b*) the intact LHRH peak can be distinguished from the degradation peak(s) produced from two separate stress conditions, and (*c*) the chemical integrity of LHRH obtained by HPLC assay correlates well with the biological potency obtained by pituitary cell culture. It is concluded, therefore, that the reported HPLC method can be used as a rapid and reliable assay.

Stability of the LHRH Solution—The HPLC chromatograms and the LHRH response curves of pituitary cultures of some representative solutions of LHRH in distilled water kept at 37°C are shown in Figs. 4 and 5 and Table III. The potency of LHRH in samples stored at 37°C for 27 weeks was reduced 10% as measured by HPLC, but showed a 40–50% decrease by bioassay between 10–27 weeks. The significant difference between the HPLC and bioassay results for the 27-week sample is real, as confirmed by reassaying samples from the same vial by both HPLC and bioassay (experiment 2, Table III). Bacterial contamination cannot be completely ruled out as a possible cause of this difference.

LHRH stored at 4°C for up to 2 years and that subjected to repeated freezing and thawing for 5 d retained 94 ± 2% and 95 ± 2%, respectively, of their chemical integrity by HPLC. These results indicate that LHRH survived for at least 10 weeks at 37°C and for at least 2 years when refrigerated. Accidental freezing and thawing causes no change in the HPLC profile, and short-term exposure to 60°C does not induce significant deterioration of the peptide hormone in distilled water as assayed by either HPLC or the biological response of pituitary cell culture. It appears that a sterile LHRH solution is adequately stable for formulation and storage either frozen or refrigerated and could be reliably used in pumps for at least 10 weeks.

Our results also demonstrate that a nonsterile solution of LHRH at ambient temperature was chemically degraded 45% in 4 months. Based on HPLC assay

Table III—Stability of LHRH in Distilled Water at 37°C

Experiment	Weeks	Potency, % HPLC	Potency, % Bioassay	
1	0	100	100	
	4	98	100	$p > 0.25$
	6	96	65	$p < 0.2$
	10	90	47	$p < 0.02$
2	0	10	100	
	10	92	66	$p < 0.2$
	27	89	60	$p < 0.05$

values of sterile LHRH solutions at 4°C or 37°C, storage for 4 months at ambient temperature of LHRH should produce little degradation. The increased degradation is probably attributable to bacterial contamination, which causes a dramatic loss of activity of polypeptides[6]. This might also explain the shorter stability of LHRH solutions observed by Dermody *et al.* (17) at 4°C (stable for 14 weeks at 4°C compared with 2 years in our experiments). They observed bacterial contamination in their solutions stored at 4°C, and the rather abrupt loss of activity reported by them between 14 and 23 weeks suggests that LHRH degradation was catalyzed by bacteria. One should, therefore, be careful in extrapolating the stability data presented in this paper to nonsterile solutions of LHRH.

Identification and isolation of degradation products was not attempted in this investigation. However, it is known that minor changes in amino acid composition of LHRH impart marked changes in agonist or antagonist bioactivity[7]. Significant differences in the bioassay and HPLC results obtained in our experiments may be due to an altered biological activity of the degradation products.

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[6] Dilute solutions (0.1–1.0 mg/mL in water) of β-endorphin (32 amino acid peptide) and corticotropine-releasing hormone (41 amino acid peptide) could lose their activity completely in 2 d at 37°C if kept nonsterile, whereas sterile solutions show little degradation (unpublished results from this laboratory).

[7] Substituting D-trypophan as the 6th amino acid of LHRH increases *in vivo* response ~100-fold.