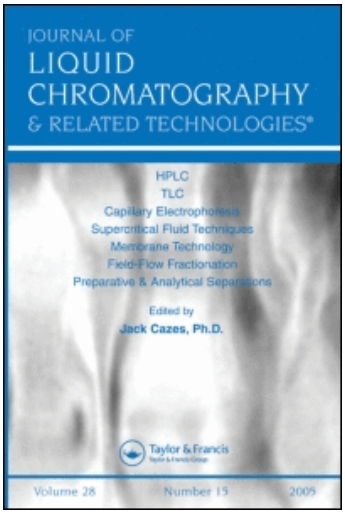

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A Valid High Performance Liquid Chromatography Method for Quantification of Luteinizing Hormone Releasing Hormone

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A VALID HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR QUANTIFICATION OF LUTEINIZING HORMONE RELEASING HORMONE

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ABSTRACT

An isocratic technique was developed for the analysis of luteinizing hormone releasing hormone (LHRH) by high performance liquid chromatography (HPLC) using 210 nm UV detection, ZORBAX ODS column (4.6 mm x 15 cm), mobile phase (86% triethylamine phosphate buffer: 14% acetonitrile), and 1.5 mL/min flow rate. The coefficient of variation (C.V.) for precision and proportionality assays was lower than 5% for all concentrations studied. The detection limit of LHRH was 1 ng/mL.

INTRODUCTION

LHRH is a decapeptide synthesized in the cell bodies of hypothalamic neurons and secreted by their terminals directly into the hypophyseal-portal blood supply.[1]

LHRH selectively stimulates the gonadotrope cells to release the heterodimeric gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), both of which stimulate gonadal production of sex steroids and gametogenesis, respectively. LHRH can be used to treat many diseases, such as endometriosis, breast cancer, precocious puberty, and prostatic cancer.[2] Termination of the action of LHRH is associated with degradation of the molecule. Several HPLC methods are now available for the analysis of LHRH.[3-5] Most of them use gradient techniques, which means that the solvent compositions change continuously during the chromatographic run. Detectors that are sensitive to changes in solvent compositions are more difficult to use with these gradient elution techniques. In isocratic technique, mobile phase (i.e., having a fixed ratio of solvents) can be more accurately controlled. Therefore, the gradient techniques are complex and baseline drift often occurs which leads to inaccurate quantification.

In this study, we developed a simple and fast separation method for the quantitation of LHRH that would be used in stability testing of the LHRH in various pharmaceutical dosage and delivery systems.

EXPERIMENTAL

Materials

Synthetic LHRH was obtained from ICN pharmaceuticals (Costa Mesa, CA). HPLC-grade triethylamine, phosphoric acid, acetonitrile, methanol, and tetrahydrofuran were obtained from Fisher (Los Angeles,Tustin, CA). C_{18} MICROSORB-MV™ column (silica 5 μm 100A, 25 cm × 4.6 mm) was from Rainin Instrument Company, Inc. (Mack Road, Woburn, MA,). ZORBAX C_8 and ZORBAX ODS columns (4.6 mm × 15 cm) were obtained from DUPONT Company (Analytical Instruments Division, Concord Plaza, Mckean Building, Wilmington, DE). All solutions and buffers were prepared with distilled deionized water.

Method

Hewlett Packard series 1050 liquid chromatograph (Hewlett Packard, Germany) was used. The above HPLC system consisted of a Pump (HP 1050), an injector (HP 1050), a variable-wavelength UV detector (HP 1050), and a computing integrator (HP 3396 A series).

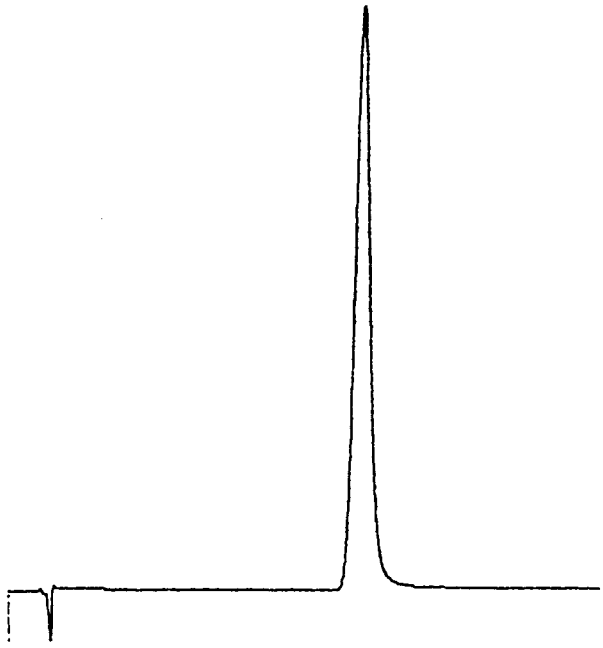

Figure 1. Standard HPLC Chromatogram of LHRH in phosphate buffer (pH 6).

LHRH and its degradation products were eluted from the column and detected at 210 nm. The mobile phase consisted of triethylammonium phosphate buffer[3] (0.36M, pH 2.5): acetonitrile (86:14 v/v). The buffer was prepared by adding 27 mL triethylamine and 18.3 mL phosphoric acid to 705.3 mL of distilled water. The injection volume was 100 μ L and the flow rate was 1.5 mL/min. LHRH stock solutions were 2 mg/mL. Test solutions were prepared by adding 25 μL of stock LHRH solution to vials containing 0.975 mL of phosphate buffer (pH 6). The reaction vial was then placed into a constant temperature oven at 80 ± 0.1°C. Samples were removed from the oven at 48 h and the concentration of LHRH was assayed by HPLC. Standard curves were constructed from five concentrations of standards (0.032-0.5 μg/μL) to determine the intra-day and inter-day variations of the method. Intra-day determinations were carried out five different times of the day. Means, standard deviations (S.D.), and coefficients of variation (C.V.) were calculated from these values.

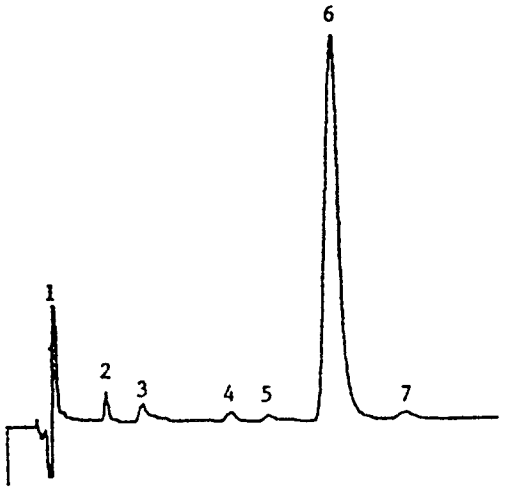

Figure 2. LHRH and its degradation products in phosphate buffer (pH 6) stored at temperature 80°C for 48 hours.

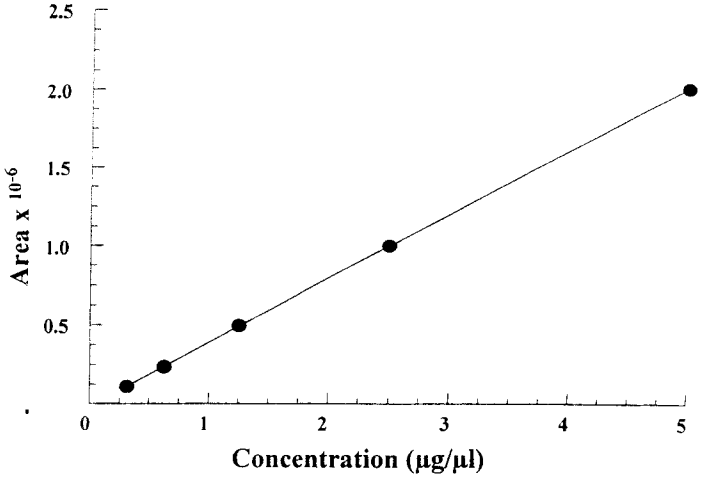

Figure 3. Standard curve of LHRH in concentrations range of 0.03-0.5 μg/μL.

RESULTS AND DISCUSSION

Optimization of HPLC Conditions

The effect of the mobile phase compositions, columns, temperature, and flow rates were investigated. Acetonitrile, methanol or tetrahydrofuran combined with triethylamine phosphate buffer (0.36M, pH 2.5) were used as mobile phase. In order to optimize the HPLC conditions for the separation of LHRH and its degradation products, three different columns were selected to optimize the resolution. We began with the mobile phase (50% acetonitrile:50% triethylamine phosphate buffer) and changed the K′ value of LHRH in C_{18} MICROSORB column by changing the flow rate and the amount of acetonitrile in the elution liquid. The main peak of LHRH was not separated from its degradation products. Also, replacement of acetonitrile with methanol and tetrahydrofuran, and restricting the K′ value within 20, did not produce satisfactory separation. The same result occurred when the column temperature was raised to 60°C.

Consequently, we changed the column to ZORBAX ODS or C_8 and kept other parameters constant. We found that ZORBAX ODS (4.6 mm ×15 cm) had the high resolution which could separate LHRH from its degradation products completely. The separation time obtained was within 15 minutes (Figures 1 and 2). Thus, we determined optimum HPLC conditions as follows: mobile phase (86% triethylamine phosphate buffer:14% acetonitrile), ZORBAX ODS column (4.6 mm × 15 cm), flow rate 1.5 mL/min, injection volume 100 μL and UV detection wavelength 210 nm.

Linearity, Detection, and Reproducibility

The linearity assay consisted of the determination of the same concentrations range of LHRH as the calibration curve (0.031- 0.5μg/μL) and each concentration was analyzed five times. The area was linearly related to the concentration for LHRH. The equation for the straight line was a = 0.40552b - 0.015 (r^2 = 0.9999) (Figure 3). The detection limit for LHRH in this method, at a signal-to-noise ratio of 3:1, was found to be 1 ng/mL. The reproducibility of the method can be expressed as both the intra-day variability and the inter-day variability. The intra-day C.V. for LHRH in the concentrations range 0.031 - 0.5 μg/μL were 0.20 - 4.55% (Table 1). Inter-day C.V. for LHRH in the same concentrations range was 0.40 - 4.63% (Table 2).

Table 1

Intra-Day Precision for LHRH Determination

Conc. (μg/μL)	Area (Five Different Times) x 10^{-6} (1)	(2)	(3)	(4)	(5)	Avg.	S.D.	%C.V.
0.5	2.010	2.010	2.010	2.010	2.020	2.012	0.0045	0.223
0.25	0.997	0.995	1.000	1.000	0.998	0.998	0.002	0.200
0.125	0.492	0.498	0.495	0498	0.499	0.496	0.003	0.604
0.0625	0.233	0.237	0.233	0.239	0.239	0.236	0.003	1.271
0.03125	0.103	0.112	0.115	0.111	0.111	0.110	0.005	4.545

Table 2

Inter-Day Precision for LHRH Determination

Conc. (μg/μL)	Area (Five Different Times) x 10^{-6} (1)	(2)	(3)	(4)	(5)	Avg.	S.D.	%C.V.
0.5	2.020	2.030	2.010	2.010	2.000	2014	0.011	0.546
0.25	0.998	0.995	0.991	1.000	0.994	0.996	0.004	0.402
0.125	0.494	0.497	0.499	0.498	0.491	0.496	0.003	0.605
0.062	0.232	0.232	0.231	0.238	0.235	0.235	0.004	1.702
0.03125	0.103	0.102	0.112	0.113	0.110	0.108	0.005	4.620

CONCLUSION

An HPLC method for quantification of LHRH was developed. The method was validated and the C.V. obtained were below the maximum permitted values. This method can be used to study the stability and quantification of LHRH in pharmaceutical dosage and delivery systems.

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