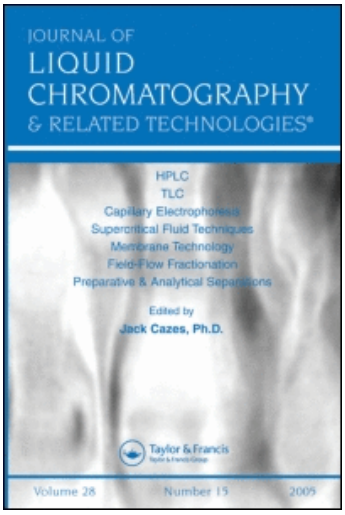

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

QUANTITATION OF LEUPROLIDE ACETATE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Jagdish Singh[a]; Sumeet K. Rastogi[a]; Som Nath Singh[a]; Jasmeet S. Bhatia[a]
[a] Department of Pharmaceutical Sciences, College of Pharmacy, North Dakota State University, Fargo, ND, U.S.A.

Online publication date: 15 November 2000

J. LIQ. CHROM. & REL. TECHNOL., 23(19), 3023–3031 (2000)

QUANTITATION OF LEUPROLIDE ACETATE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Jagdish Singh,* Sumeet K. Rastogi, Som Nath Singh, Jasmeet S. Bhatia

Department of Pharmaceutical Sciences
College of Pharmacy
North Dakota State University
Fargo, ND 58105 USA

ABSTRACT

An isocratic technique was developed for the analysis of leuprolide acetate by high performance liquid chromatography (HPLC) using 220 nm UV detection, C_{18} MICROSORB-MV™ column (4.6 mm × 15 cm), mobile phase (0.03 M, 77% dibasic ammonium phosphate buffer: 23% acetonitrile), and 2.0 mL/min flow rate.

The coefficient of variation (C.V.) for precision and proportionality assays was lower than 2% for all concentrations studied. The detection limit and tailing factor for leuprolide acetate were 100 ng/mL (signal-to-noise ratio of 3:1) and 1.16-1.26, respectively.

INTRODUCTION

Leuprolide (pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt) is a potent luteinizing hormone-releasing hormone (LHRH) agonist that stimulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), by the anterior pituitary.[1] LHRH is a decapeptide that is synthesized in the cell bodies of the hypothalamic neurons, which in turn releases the LHRH into the hypophysioportal circulation.[2]

LHRH is responsible for regulating the synthesis of LH and FSH, and causing selective stimulation of the gonadotropic cells to release the gonadotropins.[3] The gonadotropins LH and FSH stimulate the gonadal production of androgens and gametogenesis, respectively.

Leuprolide acetate can be used to treat many diseases including prostate cancer, endometriosis, precocious puberty, and metastatic breast cancer.[4] Several HPLC methods are now available for the analysis of leuprolide acetate.[5,6] Each method has its own advantages and disadvantages. Isocratic techniques show better accuracy as compared to gradient techniques, where change in the solvent compositions occur during chromatographic run, leading to complexities and baseline drift.

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

EXPERIMENTAL

Materials

Leuprolide acetate was a gift from TAP pharmaceuticals Inc. (Deerfield, IL). HPLC-grade acetonitrile was obtained from Fisher (Los Angeles, Tustin, CA). C_{18} MICROSORB-MV™ column (silica 5 μm 100 Â, 4.6 mm × 25 cm) was from Rainin Instrument Company, Inc. (Mack Road, Woburn, MA). ZORBAX C_8 (4.6 mm × 15 cm) column was obtained from DUPONT Company (Analytical Instruments Division, 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).

Leuprolide acetate and its degradation products were eluted from the column and detected at 220 nm. The mobile phase consisted of dibasic ammonium phosphate buffer (0.03 M, pH 6.4): acetonitrile (77:23 v/v). The buffer was prepared by adding 3.08 gm of dibasic ammonium phosphate to 770 mL of distilled water. The injection volume was 80 μL and the flow rate was 2.0 mL/min. Leuprolide acetate stock solutions was 0.5 mg/mL.

Test solutions were prepared by adding 120 μL of stock solution to vials containing 1.88 mL of 0.03 M phosphate buffers with different pH (3.19-7.95). The reaction vials were placed into a constant temperature oven at 90 ± 0.1°C. Samples were removed from the oven at regular intervals and the concentration of leuprolide acetate was assayed by HPLC.

Standard curves were constructed from five concentrations of standards (5-30 μg/mL) to determine the inter-day and intra-day variation of the method. Intra-day determinations were carried out at five different times of the day. Five standard samples were injected each time after a regular interval of 200 min. Five values (peak height) for each sample and five groups of data for five standard samples were calculated after 16.7 hours. The slope and r^2 for each group of data were also calculated. Means and coefficients of variation (C.V.) were calculated from these values.

Inter-day tests were carried out at the same time of each day. The calculation method was the same as that of the intra-day test. The peak asymmetry factor was calculated by $T=W_{0.05}/2f$ ($W_{0.05}$ is the width of the peak at 5% height; and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline).

Semilog graphs of the residual amounts of leuprolide acetate in phosphate buffers of various pH (3.19-7.95) versus time were plotted. The degradation rate constant (k) was obtained from the slope of the semilog plot of concentration versus time profile by statistical regression analysis.

RESULTS AND DISCUSSION

Optimization of the HPLC Conditions

The effects of mobile phase composition, columns, flow rates, and temperature were investigated. Acetonitrile or methanol, combined with dibasic ammonium phosphate buffer (0.03 M, pH 6.4), were used as mobile phase. In order to optimize the HPLC conditions for the separation of leuprolide acetate and its degradation products, two different columns were selected to optimize the resolution.

We began with the mobile phase composition of 50% acetonitrile and 50% dibasic ammonium phosphate buffer and changed the K′ value of leuprolide acetate in ZORBAX C_8 column by changing the flow rate and the amount of acetonitrile in the elution liquid. The main peak of leuprolide acetate was not separated from its degradation products. Also, replacement of acetonitrile with methanol and restricting the K′ value within 20 did not produce satisfactory operation. The same result occurred when the column temperature was raised

to 60°C. Consequently, we changed to C_{18} MICROSORB-MV™ column (4.6mm × 25 cm) and kept the other parameters constant.

We found that C_{18} MICROSORB-MV™ column had high resolution, which could separate leuprolide acetate from its degradation products completely. The separation time obtained was within 40 min. The resolution time increased when the flow rate was decreased, but the resolution factor [$R_s=1.18\ (t_2-t_1)/{}_1W_{1/2} + {}_2W_{1/2})$] did not change much and the separation time increased (t_1 and t_2: retention time of the two adjacent peaks, ${}_1W_{1/2}$ and ${}_2W_{1/2}$: peak width at half height of the peak).

Thus, we determined the HPLC conditions as follows: mobile phase (77% dibasic ammonium phosphate buffer: 23% acetonitrile), C_{18} MICROSORB-MV™ column (4.6 mm × 25 cm), flow rate 2 mL/min, injection volume 80 μL, and UV detection wavelength 220 nm. The standard HPLC chromatogram and calibration curve of leuprolide acetate are given in Figures 1 and 2, respectively.

Linearity, Detection, and Reproducibility

The linearity assay consisted of the determinations of the same concentrations of leuprolide acetate as the calibration curve (5-30 μg/mL) and each concentration was analyzed five times. The area was linearly related to the concentration for leuprolide acetate. The equation for the straight line was a = 336.624b – 38.189 (r^2 =0.9999). The detection limit for leuprolide acetate in this method, at a signal to noise ratio of 3:1, was found to be 100 ng/mL. The peak asymmetry factors for leuprolide acetate was in the range of 1.16-1.26.

The reproducibility of this method can be expressed as both the intra-day variability and inter-day variability. The intra-day system precision (%C.V.) for leuprolide acetate in the concentration range of 5-30 μg/mL was 0.23-1.96 (Table 1), and the intra-day method precision (%C.V.) was 0.612 (Table 1).

The inter-day system precision (%C.V.) for leuprolide acetate in the same concentration range was 0.17-1.55 (Table 2), and the inter-day method precision (%C.V) was 0.26 (Table 2).

Effect of pH on the Stability of Leuprolide Acetate

We also determined the separation capability of leuprolide acetate and its degradation products at different pH values (pH 3.19 – 7.9), using the above optimum conditions. As shown in Figure 3, leuprolide acetate was mostly stable between pH of 3.19 and 5.08 and only one degradation product was observed. However, leuprolide acetate degraded faster between pH 5.08-7.95, and the number of degradation products observed were increased (Figure 3).

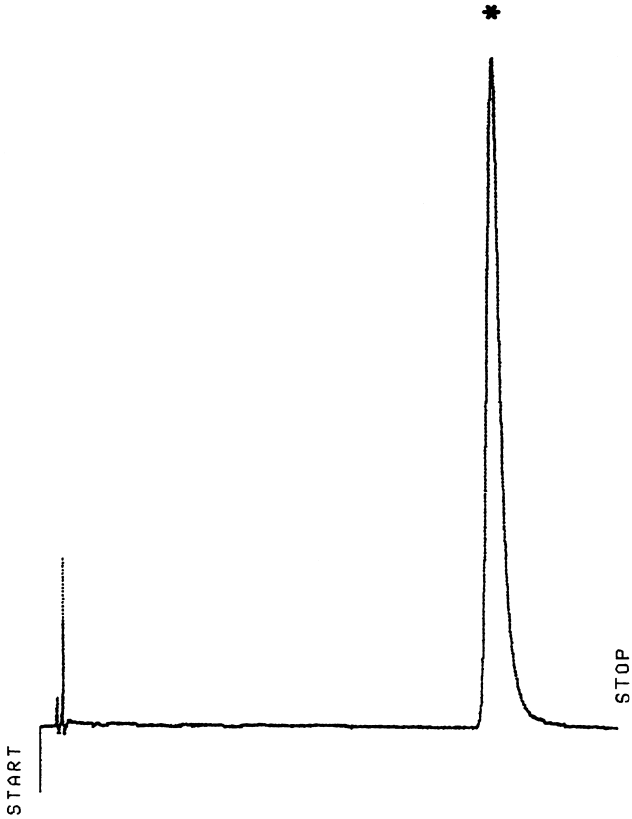

Figure 1. Standard HPLC chromatogram of leuprolide acetate in phosphate buffer (pH 3.95). * is the leuprolide acetate peak (analyzed by C_{18} MICROSORB-MV™ column).

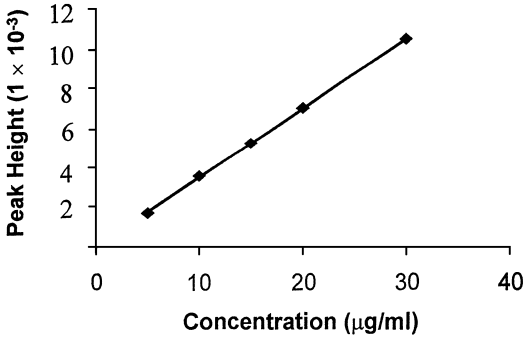

Figure 2. Standard calibration curve of leuprolide acetate in concentration range of 5-30 μg/mL.

Table 1

Intra-Day Precision for Leuprolide Acetate Determination

Conc. (µg/mL)	Peak Area 1	2	3	4	5	Average	%C.V.
30	10050	10093	10051	10086	10042	10064.4	0.231
20	6703	6742	6735	6779	6718	6735.4	0.42
15	5022	5051	5087	5046	5062	5053.6	0.46
10	3348	3351	3317	3336	3395	3341.6	1.01
5	1672	1636	1615	1649	1699	1654.2	1.96
Slope	335.794	338.154	337.567	338.159	333.348	336.604	0.612
r^2	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	

Table 2

Inter-Day Precision for Leuprolide Acetate Determination

Conc. (µg/mL)	Peak Area (Five Different Days) 1	2	3	4	5	Average	%C.V.
30	10047	10059	10071	10066	10026	10053.8	0.17
20	6710	6785	6733	6765	6705	6739.6	0.51
15	5007	5056	5071	5042	5097	5054.6	0.66
10	3357	3301	3363	3389	3322	3346.4	1.04
5	1618	1666	1687	1669	1653	1658.6	1.55
Slope	336.624	337.237	335.459	335.808	335.059	336.037	0.26
r^2	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	

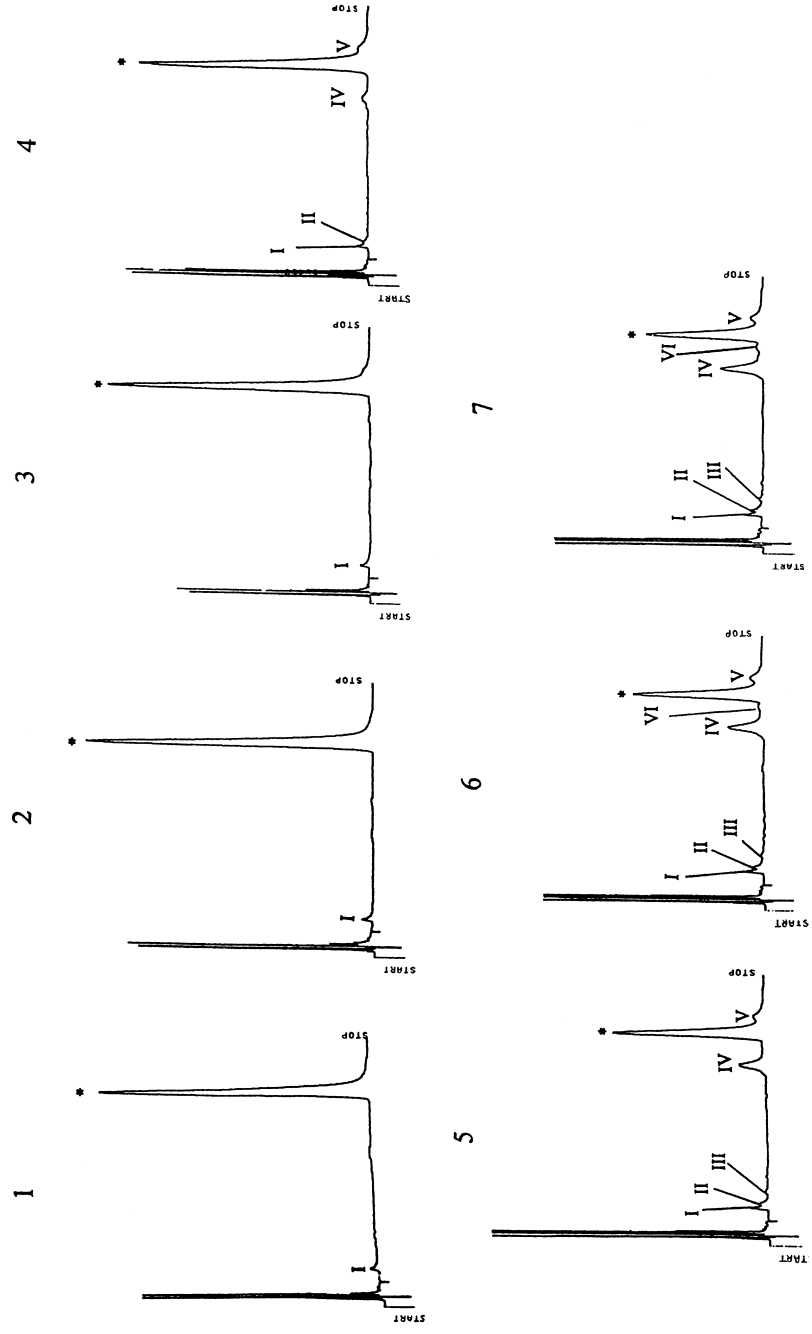

Figure 3. Leuprolide and its degradation products in different phosphate buffers (pH range from3.19-7.9) stored at temperature 90°C for 73.5 hours (analyzed by C_{18} MICROSORB-MV™ column). * is the leuprolide acetate peak. I, II, III, IV, V and VI are the degradation peaks. Keys: 1-pH 3.19; 2-pH 3.95; 3-pH 5.08; 4-pH 6.03; 5-pH 7.00; 6-pH 7.40; 7-pH 7.95.

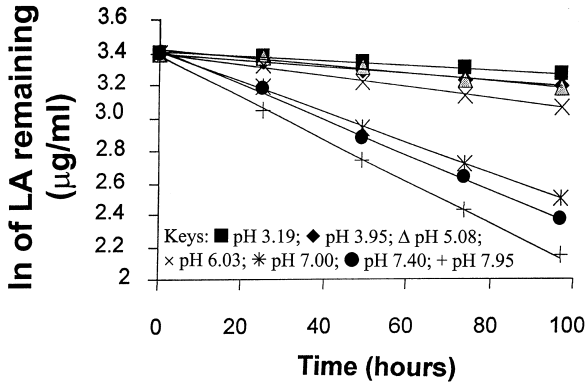

Figure 4. First order plot of the degradation of leuprolide acetate in phosphate buffer at different pH stored at 90°C.

Figure 4 shows a semilogarithmic plot of the residual amounts of leuprolide acetate versus time in phosphate buffers of varying pH at 90°C. It was found that the degradation of leuprolide acetate was pH dependent and followed apparent first order kinetics. The observed degradation rate constants (k) were obtained from the slopes of the semilog plots of concentration versus time by statistical regression analysis.

Figure 5 indicates that leuprolide acetate was most stable at pH 3.95, and the pH rate profile followed U-shaped curve. We found out that leuprolide acetate could be separated from its degradation products in each value studied.

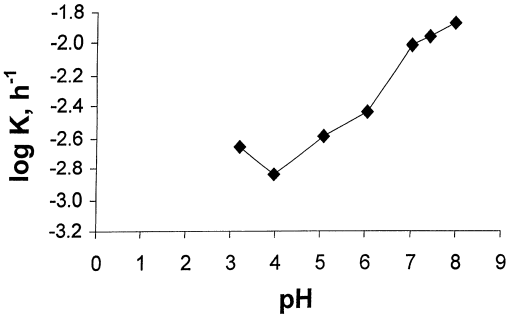

Figure 5. pH-rate profile for the degradation of leuprolide acetate at 90°C .

In this study, we have not characterized the degradation products. However, the leuprolide structure (pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt) reveals that several reaction mechanisms might occur, such as hydrolysis (HN-CO bond), oxidation, and photooxidation (His, Trp, and Tyr), and β-elimination (Ser). Therefore, the corresponding degradation products will be produced.

CONCLUSIONS

An HPLC method for quantification of leuprolide acetate was developed. The method was validated and the %C.V. obtained was below the maximum permitted values.

This method can be used to study the stability, and for the quantification, of leuprolide acetate in pharmaceutical dosage and delivery systems.

ACKNOWLEDGMENT

We gratefully acknowledge TAP pharmaceuticals Inc. (Deerfield, IL) for generously providing leuprolide acetate.

REFERENCES

1. A. L. Adjei, L. Hsu, "Leuprolide and Other LH-RH Analogues," in **Stability and Characterization of Protein and Peptide Drugs**, Y. J. Wang, R. Pearlman, eds., Plenum Press, New York, 1993, pp. 159-199.

2. P. E. Belchetz, T. M. Plant, Y. Nakai, E. J. Keogh, E. Knobil, Science, **202**, 631-641 (1978).

3. R. L. Chan, M. R. Henzl, N. E. LePage, J. LaFargue, S. A. Nerenberg, S. Anik, M. D. Chaplin, Clin. Pharmacol. Ther., **44**, 275-282 (1988).

4. A. L. Adjei, J. Garren, Pharm. Res., **7**, 565-569 (1990).

5. Y. Ogawa, M. Yamamoto, H. Okada, T. Yashiki, T. Shimamoto, Chem. Pharm. Bull., **36**, 1095-1103 (1988).

6. J. W. Sutherland, G. N. Menon. J. Liquid Chrom., **10**, 2281-2289 (1987).

Received February 8, 2000 Author's Revisions July 3, 2000
Accepted March 22, 2000 Manuscript 5257