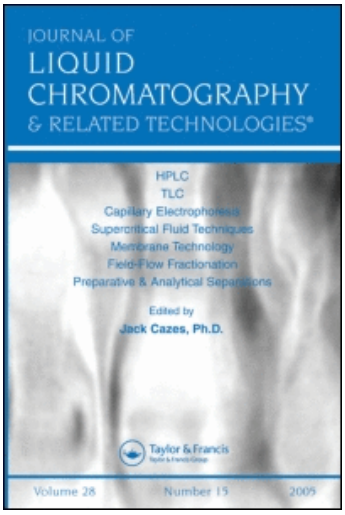

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HPLC OF LEUPROLIDE ACETATE IN INJECTABLE SOLUTIONS

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ABSTRACT

A stability indicating, HPLC method is described for the determination of leuprolide acetate in an injectable formulation. The assay is shown to be precise, accurate and sensitive to potential racemization and other degradation that could occur in aqueous solutions.

INTRODUCTION

Leuprolide acetate is a synthetic analog of leutinizing hormone-releasing hormone (LH-RH, D-Leu^{6}, L-ProNHCH$_2$CH$_3^{9}$, des-Glu NH$_2^{10}$). An injectable dosage form of the drug containing benzyl alcohol and sodium chloride has been approved by Food and Drug Administration for the treatment of prostatic cancer. Although several LH-RH analogs are being studied for their potential therapeutic effects, methodologies to assay these peptides in

dosage forms have appeared sparsely in the literature. Nishi[1], et al. have described a high performance liquid chromatography (HPLC) system used to study the racemization of various LH-RH analogs in alkaline solution. A similar HPLC system was used by Hatanaka[2], et al. to determine manufacaturing impurities in bulk leuprolide. A radioimmunoassay has been described in the literature for the assay of leuprolide and gonadotropin in sera by Yamazaki[3], et al. Sertl[4], et al. have described a reversed phase (RP) HPLC system for the analysis of the decapeptide analog of LH-RH (Schally analog), which was shown to be capable of resolving the peptide from a number of synthetic impurities and decomposition products. We describe here a RP-HPLC procedure for the assay of leuprolide in a multiple dose injectable formulation containing benzyl alcohol as a bacteriostatic agent. The HPLC system has been extensively characterized with respect to its ability to separate manufacturing impurities, potential degradation products occurring in aqueous media and several of the diastereomers.

EXPERIMENTAL

Equipment

A high performance liquid chromatograph equipped with a Waters 6000A pump, WISP injector, a multi-wavelength detector (Waters Associates, Milford, MA) and an electronic integrator/recorder (SP-4100, Spectra-Physics) was used. An octadecylsilane column (5μ), 150 mm, 4-4.6 mm i.d.) was used for the separation (IBM, Bio-Rad or Nucleosil).

Materials

All reagents and chemicals were ACS quality or HPLC grade and were used without purification. Water was triple distilled. A completely characterized lot of leuprolide acetate was used as a reference standard (Abbott Laboratories, North Chicago, IL).

Mobile Phase

The mobile phase contained 77% by volume of aqueous monobasic ammonium phosphate solution (0.087 M, pH 6.5, adjusted with ammonium hydroxide) and 23% by volume acetonitrile. The mobile phase was saturated with silica by slurrying overnight (Alltech Adsorbosil), filtered through 0.4 micrometer porosity membrane and deaerated using a magnetic stirrer and house vacuum for 2-5 minutes.

Chromatography Conditions

The flow rate was 2.0 mL/minute, the absorbance range was 0.1-0.2 AUFS at 220 nm and the injection volume was 20 microliters. All analyses were done at ambient temperature.

Internal Standard Solution

A solution containing approximately 2 mg/mL of ethyl p-hydroxybenzoate was prepared by dissolving and diluting approximately 200 mg to 100 mL with methanol.

Standard and Sample Preparations

Working standard and sample solutions were prepared by diluting together standard and internal standard solutions to give leuprolide acetate and internal standard final concentrations of 0.1 mg/mL and 0.15 mg/mL, respectively, in 0.9% w/v aqueous sodium chloride.

System Suitability

Prior to each analysis, the ability of the HPLC system to separate the degradation products was ascertained as follows. The working standard solution (5 mL) was mixed with 50 microliters of 1 N sodium hydroxide and stressed for 30-40 minutes at 100°C. The

stressed solution was neutralized with 1 M phosphoric acid and examined by HPLC. Alkaline hydrolysis under these conditions produced two major transformation products of the drug, D-Ser^4 analog which eluted prior to, and D-His^2 analog following leuprolide (Figure 1). The resolution between the D-Ser^4 and leuprolide was maintained at 1.2 or greater for consistent performance of the HPLC system.

Assay Procedure

The working standard and sample preparations were injected in duplicate into the chromatograph. The peak area ratio of leuprolide acetate to internal standard was used to calculate the concentration of the samples.

RESULTS AND DISCUSSION

The method was developed to determine the shelf-life of the formulated product and study the kinetics of degradation at various temperatures and pH values. A preliminary study of the stability of the drug in the formulation showed that the product was more stable at pH 3.3 compared to pH 10.3 when heated at 100°C for 16 hours. The recovery of the drug was almost quantitative under acid stress whereas the alkaline stress showed a potency loss of 70 percent. The alkaline stress also induced racemization of the drug to yield at least two major components, D-Ser^4 and D-His^2 leuprolide as previously reported by Nishi, et al. It was therefore decided to use the alkali stressed sample as a marker for the specificity of the system before any analysis was carried out. These isomers were detectable in aged test formulations when the pH of the formulation was $\geq$7. Glu^1-leuprolide was the major degradation product when the drug was treated with 0.1 N HCl at 40°C for 48 hours. The optimal separation of these analogs from leuprolide was obtained in the mobile phase pH-range of 6.5-7.5 and with 5 micron packings from IBM, Bio-Rad or Nucleosil.

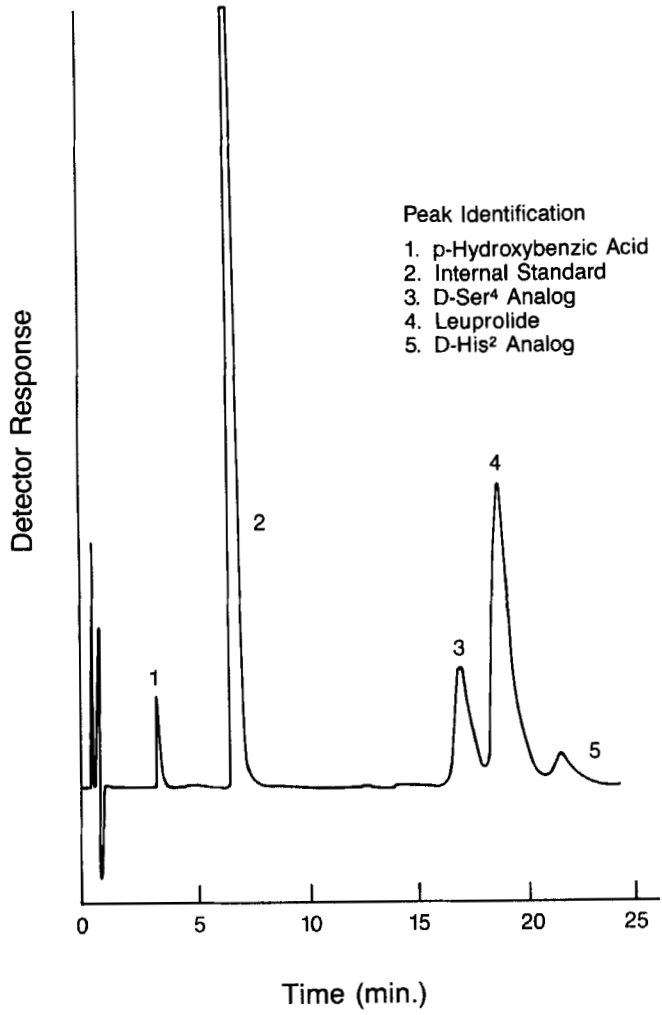

FIGURE 1. Chromatogram of a system suitability preparation.

TABLE 1

Relative Retention Times for Various Diastereomers of Leuprolide

Isomer*	Relative Retention Time
[L-Leu^6] Leuprolide	1.42
[L-Leu^6, D-Leu^7] Leuprolide	1.20
[D-Pro^9] Leuprolide	1.16
[D-Tyr^5] Leuprolide	1.15
[D-His^2] Leuprolide	1.12
[D-Leu^7] Leuprolide	1.11
[D-Trp^3, L-Leu^6] Leuprolide	1.10
Leuprolide	1.00
[D-Der^6, L-Leu^6] Leuprolide	0.88

*The isomers were synthesized during the Analog Research Program at Abbott Laboratories.

We examined several other isomers of leuprolide to assess the specificity of the proposed HPLC system. All of the isomers examined, shown in Table 1, were found to be adequately resolved under these conditions. Several of the manufacturing impurities were also separated from the drug under the same conditions. The relative retention times of these impurities with respect to leuprolide are shown in Table 2.

The response of the UV detector to varying amounts of leuprolide were found to be linear from 0.1 to 0.8)g injected on to the column (y-intercept = 0, correlation coefficient = 0.9999). Recoveries of varying amounts of the drug added to a placebo formulation are shown in Table 3. A typical chromatogram of a formulation is shown in Figure 2.

TABLE 2

Relative Retention Times for
Manufacturing Impurities in Leuprolide

Compound	Relative Retention Time
pGlu-His-Trp	0.06
[des-Leu^7] Leuprolide	0.24
Glu^1-Leuprolide	0.45
[des-ProNHEt^9] Leuprolide	0.74
Leuprolide	1.00

TABLE 3

Recovery of Leuprolide from
Addition to Formulation Placebo

Formulation Level	Percent Level of Addition	% Recovery
10 mg/mL	80	102.2
		101.2
	100	101.2
		100.6
	120	102.2
		99.7
1 mg/mL	67	102.4
	100	100.3
	133	100.2
	Mean	101.1%
Relative Standard Deviation		±1.0%

Placebo: Benzyl alcohol 10 mg/mL, sodium chloride 9 mg/mL, pH 4.4.

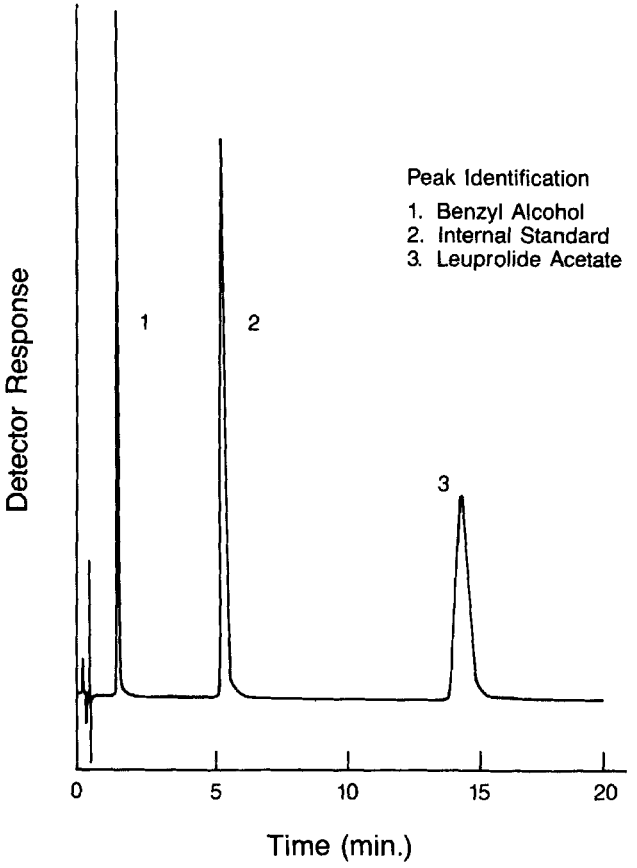

FIGURE 2. Chromatogram of a sample preparation of the formulation.

The relative standard deviation of the method determined by different analysts on 1-10 mg/mL formulation was found to be ±1.8%, n = 12.

With appropriate modifications in the sample preparation, this methodology has also been successfully used for the determination of impurities/precursors occurring during the manufacture of the bulk drug.

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