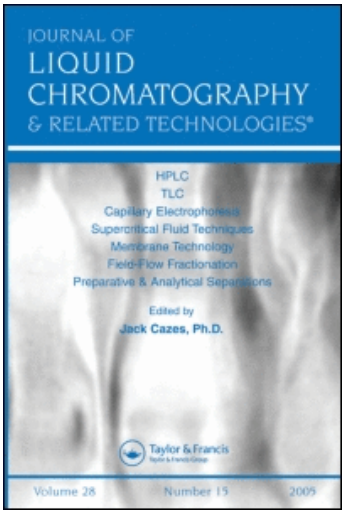

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COMPARING ELECTROCHEMICAL, FLUORESCENCE, AND ULTRAVIOLET DETECTORS FOR HPLC ANALYSIS OF THE DECAPEPTIDE, NAFARELIN

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ABSTRACT

This report describes a reverse-phase HPLC technique to determine the concentration of nafarelin (a decapeptide luteinizing hormone-releasing hormone analog) in aqueous solutions for intranasal administration. Pursuant to the method development we evaluated three different detectors with respect to sensitivity, linearity, specificity and reliability. The three detector types investigated were: spectrophotometric (225 nm), electrochemical (at +1.2 v), and fluorescence (excitation = 282 nm, emission = 332 nm). All three detectors gave satisfactorily linear response, and gave equivalent results for nafarelin samples assayed in parallel. The lower detection limits for the three detectors were: ultraviolet = 1.5 ng, electrochemical = 2.0 ng, and fluorescence = 0.6 ng. Thus, the three detector types are

nearly equally sensitive for nafarelin analysis. For routine determinations the ultraviolet detector is superior to the electrochemical and fluorescence detectors with respect to convenience of operation.

INTRODUCTION

Nafarelin (Figure 1) is a decapeptide analog of luteinizing hormone-releasing hormone with therapeutic indications for prostatic carcinoma and endometriosis (1). While developing a reverse-phase HPLC technique to assay nafarelin concentrations in aqueous solutions for intranasal administration, we considered the relative advantages and disadvantages of different detector

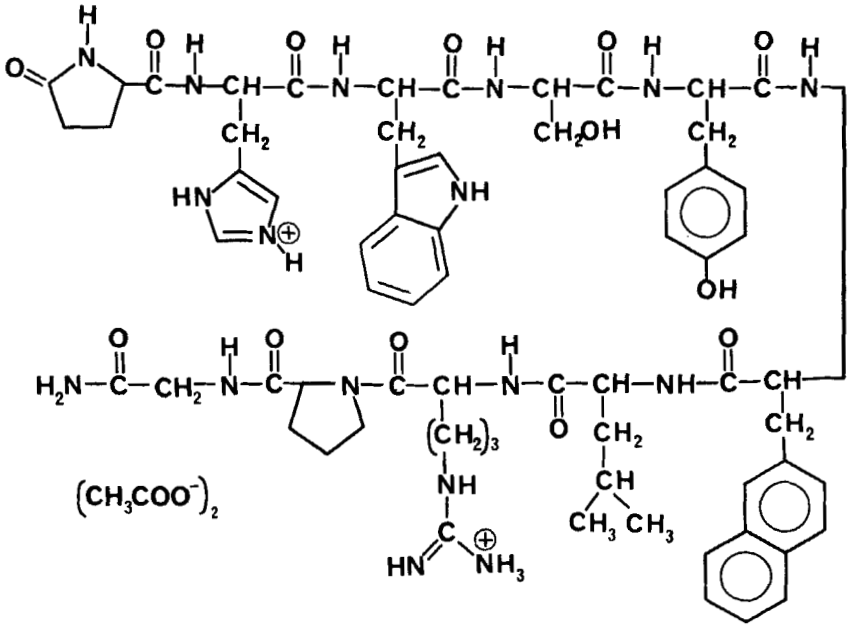

Figure 1. Structural Representation of the Decapeptide, Nafarelin.

types. Nafarelin features naphthyl, tyrosyl, and tryptophyl functional groups and consequently responds well to ultraviolet, electrochemical, and fluorescence detectors. Although the literature refers most frequently to ultraviolet detection for peptides and proteins, electrochemical (2-8) and fluorescence (9-12) techniques also find application for sensitive and specific peptide analysis. Some literature references directly compare two detection techniques (i.e. electrochemical versus ultraviolet or fluorescence versus ultraviolet) but we were unable to identify any head-to-head performance tests of all three detection types for a single peptide.

Considering the foregoing we undertook to compare electrochemical, ultraviolet, and fluorescence HPLC detectors with respect to suitablity, selectivity, and sensitivity for nafarelin analysis. Our investigation included the following determinations:

1) spectroscopic and voltammetric characteristics of nafarelin and its hydrolysis products,

2) equivalence of parallel assays with tandem ultraviolet, electrochemical and ultraviolet-fluorescence detectors,

3) linearity and lower detection limit, and

4) the HPLC detector "figure of merit" suggested by Roe (13).

We find that all three detector types perform satisfactorily, the fluorescence detector giving somewhat higher sensitivity than the electrochemical and ultraviolet detectors. However, the detection limits for the three detector types are not greatly different and convenience recommends ultraviolet detectors for routine nafarelin analysis.

EXPERIMENTAL DETAILS

Materials.

Nafarelin acetate and authentic samples of two hydrolysis products (the 1-9 peptide free acid and the 4-10 peptide amide) were prepared by the Syntex Institute of Organic Chemistry according to the published (1) procedure. The drug is formulated at 2 mg/mL in an isotonic aqueous solution for nasal administration. Acetonitrile and methanol (Burdick and Jackson Labs) were HPLC grade. Dibutyl amine (Eastman Kodak), potassium dihydrogen phosphate (Mallinkrodt) and phosphoric acid (Mallinkrodt) were used as obtained from the suppliers. Distilled water for mobile phase and sample preparation was further purified with a Barnstead Nanopure filtration system.

HPLC System.

The following components comprised the HPLC instrumentation: model M6000A pump (Waters Assoc.), model 748C column oven (SpectraPhysics), model 710B WISP autosampler (Waters), and model SP4000 integrator (Spectra-Physics). The analytical column was a 4.6 x 250 mm, 5 μm particle size, 300 °A pore size, C18 "Protein and Peptide" column (Vydac). A 2.1 x 70 mm column packed with CO:Pell ODS (Whatman, Inc.) protected the analytical column.

Mobile phase was a 23.2:76.8 (v:v) mixture of acetonitrile with 25 mM dibutyl ammonium phosphate plus 25 mM potassium dihydrogen phosphate adjusted to pH = 3.0 with phosphoric acid. With 1.0 mL/min solvent delivery and 45 °C column temperature, the backpressure was approximately 1000 psi.

Detectors.

Ultraviolet spectra were recorded with a model 1040A diode array detector (Hewlett-Packard). HPLC traces were recorded with LDC Spectromonitor III variable wavelength detector at 225 nm.

A model LC-4B (Bioanalytical Systems) detector with glassy carbon working electrode at +1.2 volts versus Ag/AgCl reference electrode was used for electrochemical detection. Cyclic voltammograms were recorded with a model EC-225 voltammetric analyzer (IBM Instruments) using glassy carbon working electrode, Ag/AgCl reference, and platinum wire auxiliary electrodes.

Excitation and emission spectra were obtained with a model FL-749 (McPherson) spectrofluorometer equipped with a model 7898 scan controller, a xenon lamp and 1x1 cm cuvettes. HPLC detection used: a 24-μL flow cell, a model UT excitation filter, a model CF-320 flow cell filter, excitation wavelength = 282 nm, and emission wavelength = 332 nm. For comparison, we also examined a deuterium lamp with no filters and set at excitation = 239 nm and emission = 270 nm.

Procedures.

Sample preparation required diluting an aliquot of nasal solution to 4 μg/mL final concentration in a 25:75 mixture of methanol with aqueous 25 mM potassium dihydrogen phosphate. Injection volumes were 40 μL and peak height quantitation was used throughout.

Nafarelin solutions were partially hydrolyzed to produce degradation products by maintaining nasal formulation samples at 80 °C for 5 d. These samples showed approximtely 80% nafarelin remaining.

Ultraviolet spectra were recorded in water, fluorescence spectra, and cyclic voltammograms were obtained in a 25:75 mixture of methanol with 25 mM potassium dihydrogen phosphate.

Method linearity and lower detection limit for each detector were determined by assaying solutions spiked with 0.5 to 22 % of 2 mg/mL nafarelin. For each detector we plotted nafarelin peak heights versus ng of nafarelin injected and determined linear least-squares regression slopes and intercepts. We define the detection limit as the intersection of the lower 95% confidence interval about the peak height versus ng injected slope with a peak height value equal to twice the baseline noise level.

We also assessed detector sensitivity by determining the "figure of merit" described by Roe (13). For each detector we measured nafarelin peak response (S), baseline noise (N), and peak width at half height (W). For a given nafarelin injected amount (ng) and injection volume (V), the figure of merit equals the ratio, (S*V)/(N*W*ng), and has units of reciprocal nanograms. The figure of merit numerical value thus is inversely proportional to detector sensitivity.

RESULTS AND DISCUSSION

Nafarelin Spectral and Electrochemical Characteristics.

We recorded ultraviolet and fluorescence spectra and cyclic voltammograms of nafarelin and its hydrolysis products. Our objectives in this regard were threefold. First, we wished to identify appropriate spectral and electrochemical parameters for HPLC detection. Secondly, the spectral and voltammetric data establish which functional groups present in the parent molecule

Table I.
Ultraviolet Spectral Absorbance Maxima For Nafarelin and Related Compounds.

Compound	Solvent	Absorbance Maxima	
		Wavelength nm	Extinction Coeff. 1000/M-cm
Nafarelin	Water	225	127
		276	11.5
Naphthalene*	EtOH	221	110
		275	5.8
Tyrosine*	Water	275	1.4
Tryptophan*	Water	280	6.3

* Data of reference 14.

contribute principally to observed ultraviolet, fluorescence, and electrochemical responses. Finally, to the degree that nafarelin and its degradation products feature different spectral and voltammetric responses, the three detector types investigated provide additional specificity for nafarelin HPLC analysis.

Table I summarizes ultraviolet spectral characteristics for nafarelin and related compounds. The absorbance maxima for naphthalene closely approximate those of nafarelin, both with respect to wavelength and extinction coefficient. By comparison, neither

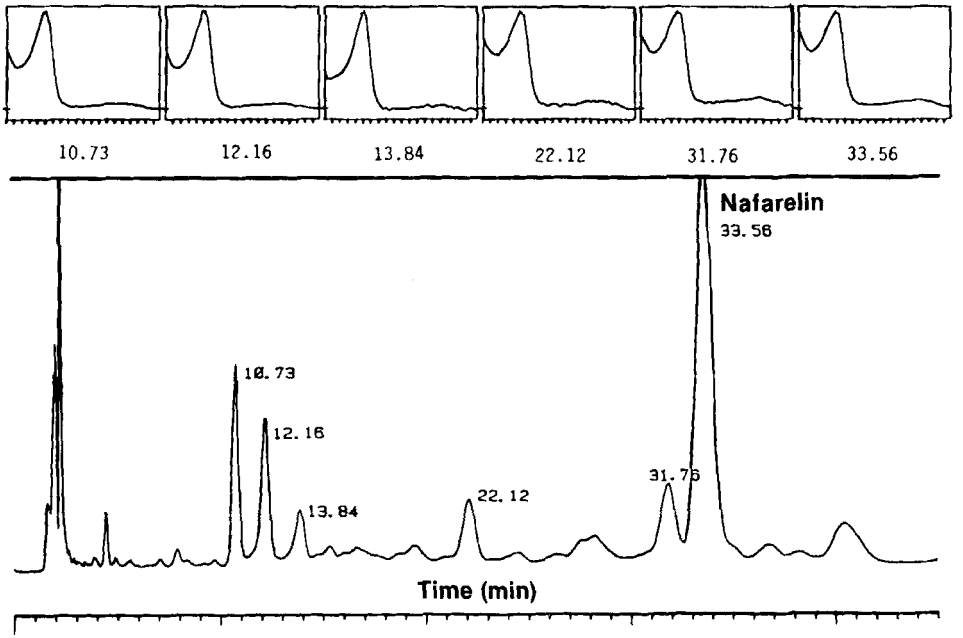

Figure 2. Representative Chromatogram of a Partially-Hydrolyzed Nafarelin Nasal Solution Sample (160 ng Nafarelin on column). The Spectra at the Top of the Chromatogram Are Indexed to the HPLC Peaks With the Indicated Retention Times.

tyrosine nor tryptophan absorb maximally in the 200 to 270 nm range. Thus, the naphthyl moiety provides the intense low wavelength absorbance band characteristic of nafarelin.

We also obtained spectral data for nafarelin degradation products by chromatographing a partially-hydrolyzed nasal solution sample and acquiring the spectra of degradation product peaks "on-the-fly" with a diode array detector. Figure 2 shows a representative chromatogram of a degraded sample with spectral scans for

the major HPLC peaks (excluding the solvent artifact). From the figure, it is evident that all of the HPLC peaks examined gave essentially identical spectra. Thus, ultraviolet detection provides no additional specificity for nafarelin HPLC analysis.

Figure 3 shows nafarelin fluorescence emission and excitation spectra. The maxima appeared at 282 nm (excitation) and 332 nm (emission). Fluorescence spectra of two nafarelin hydrolysis products (the 1-9 peptide free acid and the 4-10 peptide amide) were superimposable with parent molecule spectra.

Literature (15,16) excitation and emission maxima for related compounds are as follows:

naphthalene excitation = 275 nm and emission = 335,
tyrosine excitation = 280 nm and emission = 354, and
tryptophan excitation = 280 nm and emission = 303 nm.

Comparing the emission maximum for nafarelin with the maxima for naphthalene, tyrosine, and tryptophan suggests that nafarelin fluorescence resides principally in the naphthyl moiety with relatively minor contributions from the tyrosyl and tryptophyl groups.

Figure 4 is a cyclic voltammogram of a 0.100 mg/mL nafarelin solution. The voltammogram reveals a single anodic wave with peak potential at +1.1 v. The absence of a cathodic wave on the reverse scan indicates that nafarelin oxidation is electrochemically irreversible. The 1-9 peptide free acid and the 4-10 peptide amide fragments gave cyclic voltammograms essentially identical to the one seen in Figure 3. Because the naphthyl moeity does not oxidize at accessible potentials in aqueous methanol, we assign the electrochemical activity of nafarelin to the tryptophyl and tyrosyl functional groups.

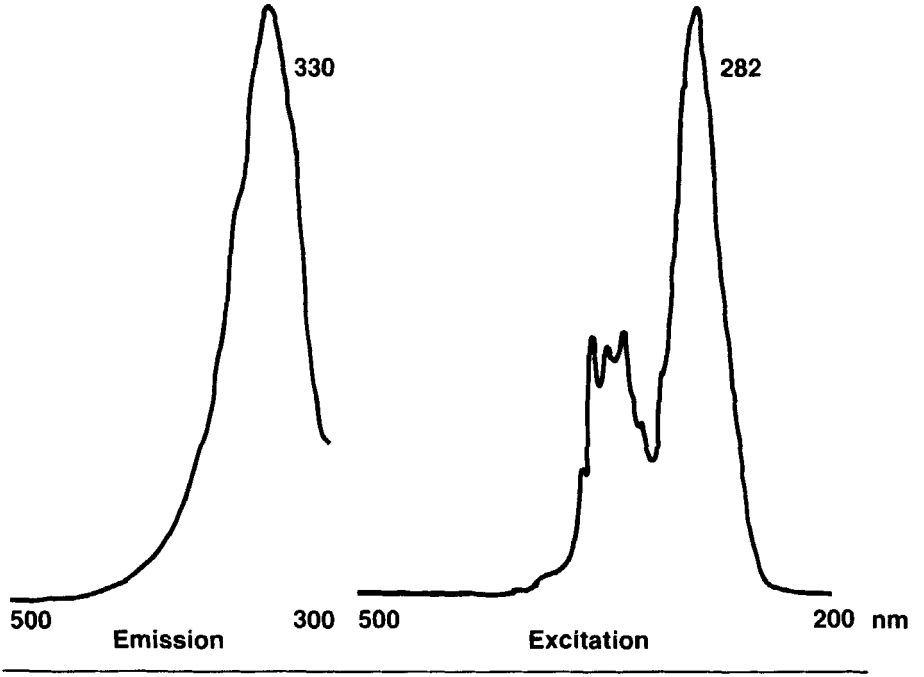

Figure 3. Fluorescence Spectra of Nafarelin in Aqueous Methanol.

Detector Performance.

Figure 5 shows representative chromatograms obtained with ultraviolet, electrochemical and fluorescence detectors.

To demonstrate that the different detector types give identical quantitative results for nafarelin assays, we connected the ultraviolet detector in series with the fluorescence detector (ultraviolet detector upstream). We then assayed five different partially-hydrolyzed (60 to 95 % remaining) nafarelin solution

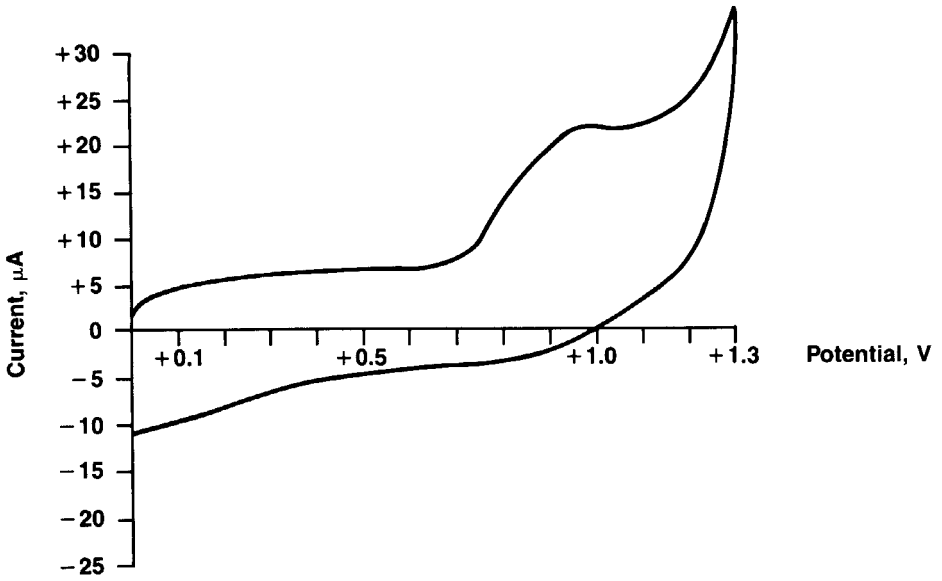

Figure 4. Cyclic Voltammogram of Nafarelin in Aqueous Methanol.

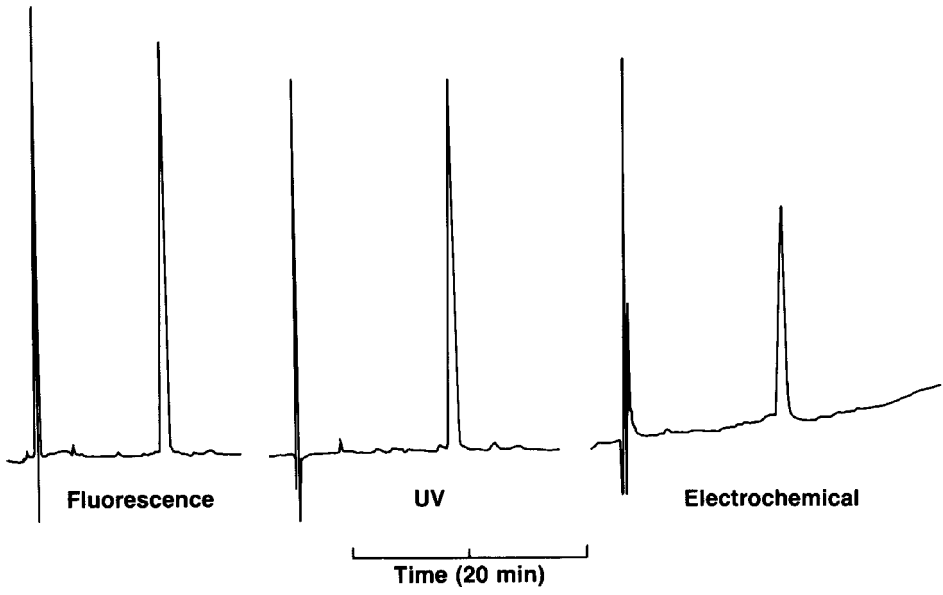

Figure 5. Representative Chromatograms of Nafarelin Nasal Solutions Using Ultraviolet, Electrochemical, and Fluorescence Detectors.

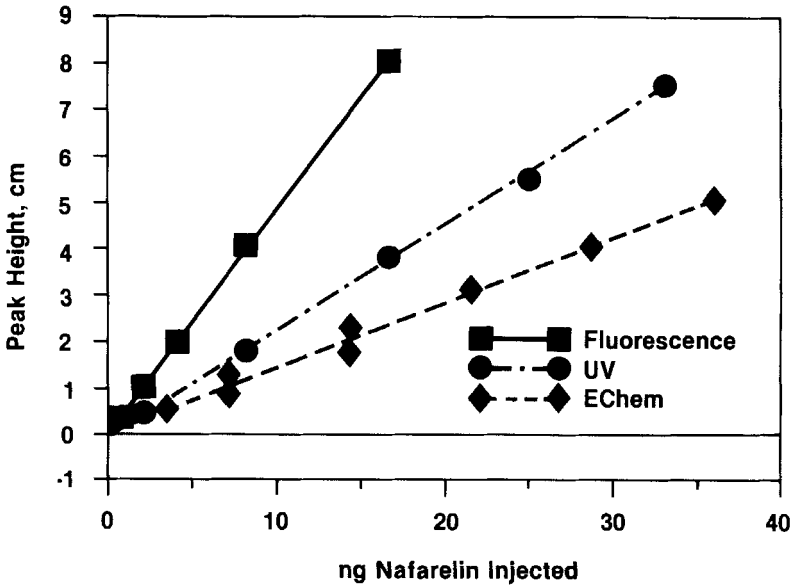

Figure 6. Response Linearity and Lower Detection Limit Plot for Nafarelin Determinations by Ultraviolet, Fluorescence, and Electrochemical Detectors.

samples. For the five samples the ratio, [nafarelin by fluorescence detection]÷[nafarelin by ultraviolet detection] averaged 1.002 ± 0.007. We repeated the determinations of the five samples with tandem electrochemical/ultraviolet detection and the assay ratios averaged 1.007 ± 0.013.

Figure 6 is a plot of HPLC peak height versus ng nafarelin injected for placebo solutions spiked at 0.5 to 22 % of 2 mg/mL and assayed in duplicate using the three different detector types. Table II summarizes the linearity and lower detection limit statistics for these determinations. From the table, it is

Table II.
Linearity and Lower Detection Limit Statistics
for Nafarelin Analysis.

Detector	Lower Detect. Limit ng	"Figure of Merit" 1/ng	Linearity Slope* mm/ng	Linearity R
Ultraviolet	1.5	0.23	2.29 ± (0.04)	0.999
Electrochem	2.0	0.06	1.41 ± (0.04)	0.998
Fluorescence	0.60	0.38	9.65 ± (0.16)	0.999

* Values in parentheses are least-squares ± 95 % confidence intervals.

evident that all three detectors give excellent response linearity over the concentration range investigated. The lower detection limits and the "figure of merit" values both show that fluorescence detection is the most sensitive of the three techniques investigated. Not shown in the table are sensitivity data for fluorescence detection using a deuterium lamp in place of the xenon lamp. The deuterium lamp gave a 2.0 ng lower detection limit, and therefore is significantly less sensitive than the xenon source.

CONCLUSIONS

Fluorescence, electrochemical and ultraviolet detectors quantitate nafarelin solutions with equivalent linearity and accuracy. The three detection techniques are also alike insofar as they do not provide any unique spectral or electrochemical signatures that might distinguish the drug from its hydrolytic degradation products.

The fluorescence detector (with xenon lamp) was more sensitive than the ultraviolet detector by a factor of 2.5, whereas the ultraviolet and electrochemical detectors are essentially equally sensitive.

When the highest sensitivity is required, the fluorescence detector has some application for nafarelin determinations. For routine analysis of nafarelin nasal solutions, however, the combined excellent reliability and good sensitivity recommend ultraviolet detection.

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