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Degradation of the LH-RH analog nafarelin acetate in aqueous solution

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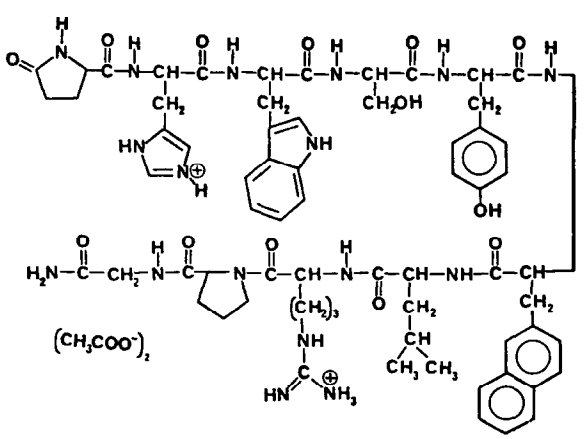
Summary

The degradation of the superagonist LH-RH analog nafarelin acetate (I) in aqueous solution was followed by reverse-phase HPLC. The log k_{obs} vs pH profile for I at 80°C displayed a U-shaped profile with a pH region of maximum stability between pH 4 and 6. In the acid pH region, specific acid-catalyzed hydrolysis of the glycinamide group occurs to give the free acid of I as a major degradation product. HPLC chromatograms indicate that numerous products, including a small amount of free acid, are formed in the region from pH 4 to 10. Degradation at lower temperatures indicate the pH of maximum stability was pH ~ 5.3 at 40°C with a predicted T_{90} of 3.8 years at 25°C.

Introduction

The decapeptide nafarelin acetate (I; [D-Nal(2)^6]LH-RH) is a superagonist analog of the naturally occurring hormone LH-RH (Nestor et al., 1982). This synthetic peptide is being investigated as a contraceptive agent and for a variety of other hormone-dependent conditions (Vickery et al., 1984; Bex and Corbin, 1984). Since peptides are not generally orally active, aqueous formulations for parenteral and nasal administration of I were developed for use in clinical trials (Anik et al., 1984). While there have been some reports on the stability of LH-RH or its analogs (Nishi et al., 1980; Feldman et al., 1978; Sertl et al., 1981; Winterer, 1983), there is little fundamental information on the chemical stability of these important peptides in the pH region of interest for



Nafarelin (I)

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formulation development. We have therefore investigated the kinetics of degradation of I as a function of pH by HPLC.

Experimental

Materials

The nafarelin acetate (I; Nestor et al., 1982) and its free acid were obtained from the Institute of Organic Chemistry, Syntex Research. The acetonitrile used was HPLC grade and the water was purified through a filtration and ion exchange system [1]. All other chemicals were reagent grade.

HPLC Methods

The HPLC system consisted of a pump [2], column oven [3], autosampler [4], and variable wavelength detector [5]. Nafarelin and its degradation products were detected at the λ_{max} in mobile phase, 225 nm. At this wavelength, the relative absorbance of the D-Nal, Tyr and Trp residues is 46 : 5 : 1, respectively. Thus, only degradation products containing D-Nal or Tyr will be evident in the chromatogram. The response factors for nafarelin and its free acid were essentially identical at this wavelength. The column was heated to 40°C to provide lower back pressure, shorter retention times and an increase in assay precision compared to ambient temperature. Quantitation by peak height or peak area integration [6] gave excellent linearity up to 3 μg injected.

Method A

This method was used to quantitate nafarelin in all the kinetic studies. It utilized an ODS column [7] and a mobile phase of 0.195 M monobasic potassium phosphate buffer (pH ~ 4.4)–acetonitrile (26.5 : 73.5). At a flow rate of 1.8 ml/min, a pressure of 3400 psi developed using this method. Typically, 50 μl of sample was injected. The chromatograms of a standard and degraded samples of

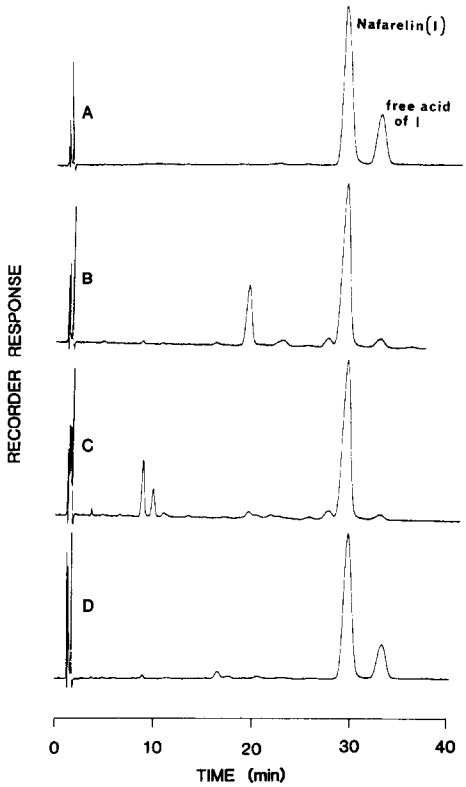

Fig. 1. HPLC chromatogram using Method A. A: standard solution of nafarelin and its free acid. B: pH 10.52 solution. C: pH 5.43 solution. D: pH 0.96 solution. Solutions B, C and D were degraded at 80°C and all have ~ 70% nafarelin remaining.

I are shown in Fig. 1 using Method A. The specificity of this method for I in the presence of its degradation products is indicated by the first-order decrease in peak height (or area) with increasing degradation time at all pHs. Further evidence of specificity was that identical concentration values of I were obtained for highly degraded nafarelin samples (as low as 15% I remaining) when an independent HPLC method was used (Method B described below).

Method B

This method used an ODS column [8] and a mobile phase of 0.025 N N,N-dimethyloctylammonium phosphate buffer (pH3.0)–acetonitrile (79.5 : 20.5). A pressure of 2000 psi developed on

[1] Barnstead Nanopure System.
[2] Spectra-Physics Model 3500.
[3] Spectra-Physics Model 748.
[4] Micromeritics Model 725 with 10 μl loop.
[5] Kratos Model SF-770.
[6] Spectra-Physics Model SP-4000.
[7] Beckman Ultrasphere I.P. 5-μ ODS column (25 cm×4.6 mm i.d.).
[8] Beckman Ultrasphere 3-μ ODS column (7.5 cm×4.6 mm i.d.).

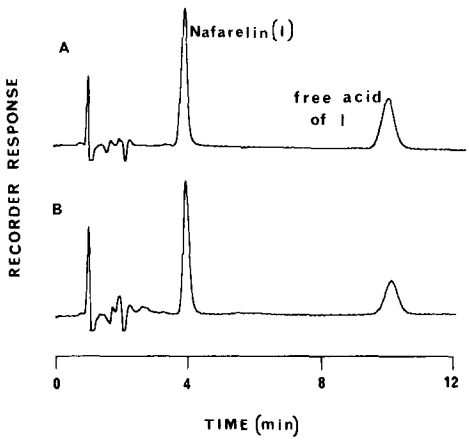

Fig. 2. HPLC chromatogram using Method B. A: standard solution of nafarelin and its free acid. B: solution degraded at pH 0.96, 80°C with ~ 70% nafarelin remaining.

this method at a flow rate of 1.0 ml/min at room temperature. An example chromatogram of a nafarelin standard and degraded solution using Method B is shown in Fig. 2.

Preparation of standards

Due to the hygroscopic and electrostatic nature of the amorphous solid, all weighing operations were performed at constant relative humidity. Static charge was reduced at higher humidities, thus the peptide was equilibrated for at least 1 day in a 79% relative humidity chamber (a dessicator containing a saturated aqueous ammonium chloride solution) prior to weighing.

It was found that water gain or loss was rapid (~ 1 min) when solid I was exposed to changes in humidity. Exposure to room humidity was thus avoided during weighing operations by capping weighing containers prior to removal from the relative humidity chamber. The equilibrium moisture content as a function of humidity is shown below for lyophilized peptide:

Relative humidity	Water content in I (%)
47	3.3
68	5.0
79	7.0
93	12.5

Kinetic methods

Buffer solutions contained 0.020 M total buffer and KCl was added to make the ionic strength 0.15 at room temperature. The solutions at the pH extremes employed HCl and KOH to adjust the pH. The pH of all solutions was measured at the appropriate temperature (40–80°C) using a combination electrode [9].

Solutions were typically prepared by stirring a mixture of 2.7 mg of solid drug in 50 ml of buffer solution for 1 h followed by filtration through a 0.22 μm membrane [10] to give a final drug concentration of 55 μg/ml (4.0×10^{-5} M). At this concentration in the presence of buffers, no drug adsorption to glass occurred [11]. The solutions were then sealed in glass ampules and placed in a constant temperature bath at the appropriate temperature (40–80°). The solutions were removed after the appropriate period of time and stored at 4°C until they were assayed. The solutions were diluted 1:1 with mobile phase and analyzed by HPLC for the amount of I remaining.

Results and Discussion

The degradation rate of nafarelin acetate (I) was studied in aqueous solution from 40°C to 80°C and at constant pH. Disappearance of I was followed using a reverse-phase HPLC method. At the pH extremes, the degradation reaction obeyed pseudo-first-order kinetics through three half-lives. Fig. 3 shows an example first-order plot of the rate data obtained at pH 0.96 and 80°C. The degradation of I in the intermediate pH region and at lower temperatures was followed to less than one half-life in some cases due to the slowness of the reaction. At 40°C, degradation of I was followed for 2 years. The observed pseudo-first-order rate constants (k_{obs}) are listed in Table 1.

The log k_{obs} vs pH profile for the degradation

[9] Radiometer Model PHM 64 pH meter with Radiometer Model GK2401C electrode.

[10] Metricel GA-8 membrane.

[11] Adsorption to glassware can occur in pure water solutions at low drug concentrations (Anik and Hwang, 1983).

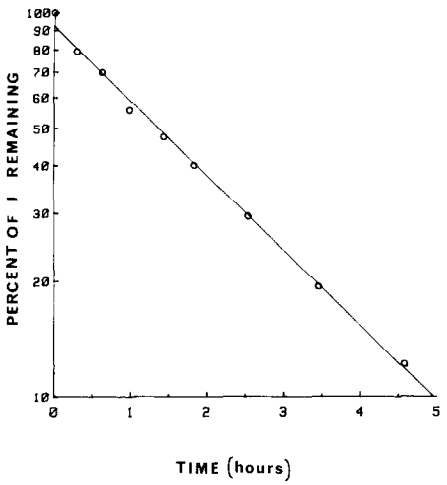

Fig. 3. First-order plot for the degradation of nafarelin at pH 0.96 and 80°C.

TABLE 1

OBSERVED RATE CONSTANTS FOR THE DEGRADATION OF NAFARELIN [a]

pH [b]	Temp. (°C)	Buffer [c]	$k_{obs} \times 10^6$ (s^{-1})
0.96	80	HCl	125
2.06	80	HCl	8.51
3.25	80	Formate	0.80
3.95	80	Acetate	0.30
4.54	80	Acetate	0.17
5.43	80	Acetate	0.24
5.77	80	Phosphate	0.32
7.40	80	Phosphate	2.3
9.21	80	Carbonate	59
10.52	80	KOH	662
4.62	60	Acetate	0.035
5.43	60	Acetate	0.038
6.25	60	Succinate	0.048
4.60	50	Acetate	0.014
5.40	50	Acetate	0.012
6.22	50	Succinate	0.017
4.57	40	Acetate	0.0069
5.09	40	Acetate	0.0066
5.37	40	Acetate	0.0054
6.17	40	Succinate	0.0086

[a] Nafarelin acetate concentration was 55 μg/ml (4×10^{-5} M).
[b] pH was measured at the temperature indicated. The pH 0.96 solution was calculated from the data of Greely (1960).
[c] Buffer concentrations 0.020 M except for HCl and KOH solutions which were 0.15 M HCl (pH 0.96), 0.01 M KOH (pH 10.52). KCl was added, where needed, to adjust the ionic strength to 0.15

of I at 80°C is defined by the open circles in Fig. 4. The data points are connected by line segments to illustrate the U-shaped curve of the profile. The acid portion of the profile between pH 1 and pH 3 exhibits a slope of −1 indicating the reaction is specific-acid catalyzed. In this pH region, a major degradation product was identified by retention time on two HPLC methods as the free acid of I (Figs. 1 and 2). Based on HPLC analysis of a standard of the free acid, approximately 65% of the degradation products can be accounted for in this pH region by this product. Thus, acid hydrolysis of the glycinamide group in I is the predominant reaction occurring below pH 3 with a second-order rate constant $k_h \sim 1.0 \times 10^{-3}\ s^{-1} \cdot M^{-1}$ at 80°C. Values of k_h for a terminal glycinamide group in other peptides has not been reported. However, the acid-catalyzed hydrolysis of acetamide, the simplest model for the glycinamide group in nafarelin, has been studied from 60°C to 75°C by T. Yamana and co-workers (1972). Using their second-order rate constants and activation energy value, k_h for acetamide at 80°C was calculated to be $\sim 1.3 \times 10^{-3}\ s^{-1} \cdot M^{-1}$, nearly identical to that found for I.

The remainder of the pH profile in Fig. 4 is comprised of the shallow curved area from pH 4 to pH 6 and the base-catalyzed segment, exhibiting a slope of ~0.8. The complex nature of the

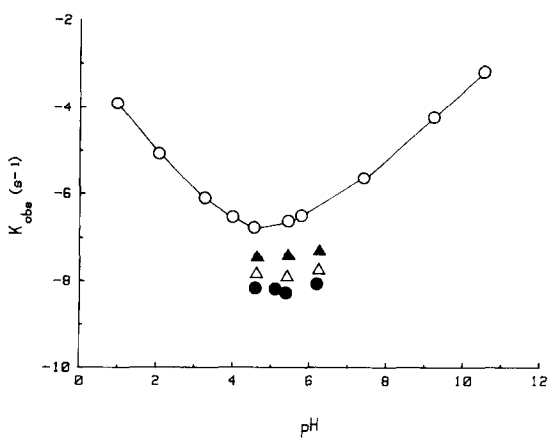

Fig. 4. Log k_{obs} versus pH profile for the degradation of nafarelin in aqueous solution at 80°C (○), 60°C (▲), 50°C (△), and 40°C (●). The data points are connected by line segments to show the shape of the profile.

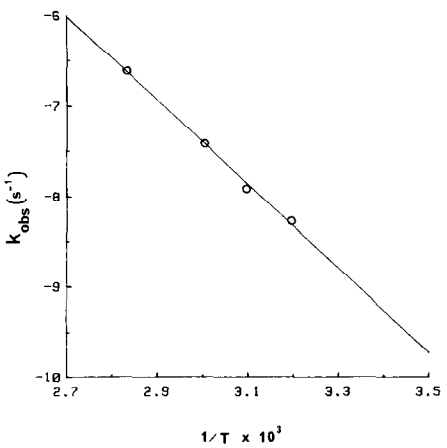

Fig. 5. Arrhenius plot of log k_{obs} vs. 1/T for the degradation of nafarelin in aqueous solution at pH 5.4.

degradation reactions in this region is mirrored in the chromatograms shown in Fig. 1. At both pH 5.4 (Fig. 1C) and pH 10.5 (Fig. 1B) several degradation products are seen in the chromatograms. With the exception of a small amount of free acid, the products formed in this pH region have not been identified. D-Ser[4]-I is a likely product at high pH (pH 10.5) since racemization of Ser[4] was shown to occur in a similar LH-RH analog by Nishi and co-workers (1980). The major degradation products of I appear to be relatively stable since their concentration increases as the degradation of I progresses. These observations support the idea that I may degrade by several independent pathways from pH 4 to 10[12].

Degradation of I at 60, 50 and 40°C was examined in the pH region 4.5–6.2 where the peptide is most stable. The rate constants are listed in Table 1 and are plotted in Fig. 4. From Fig. 4, the pH of maximum stability shifts somewhat with temperature and is ~ pH 5.3 at 40°C. An Arrhenius plot of the data at pH 5.4 is reasonably linear (Fig. 5) and the slope gives an activation energy for the overall reaction of 21 kcal/mol. Extrapolation to 25°C gives a predicted mean T_{90} of 3.8 years and a minimum T_{90} of 1.9 years using 95% confidence intervals on the data from the Arrhenius plot.

[12] Construction of an equation (or equations) to fit the data in this pH region is possible, considering that there are three ionized species present (imidazole and tyrosine pK_as 5.9 and 9.9 at 25°C), each with its own reactivity as a function of pH. However, this exercise is not meaningful without knowledge of the major degradation products and pathways in this pH region.

Acknowledgements

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