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Report

Stability of Gonadorelin and Triptorelin in Aqueous Solution

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The influence of pH, temperature, various buffer species at different concentrations, and ionic strength on the stability of gonadorelin and triptorelin in aqueous solution has been studied using stability-indicating high-performance liquid chromatographic methods. The degradation behavior of both peptides is similar. The maximum stability of both peptides was shown to be at an approximate pH of 5.0. Acetate has the most favorable effect on stability, while phosphate causes higher degradation. Varying the concentration of acetate buffer does not affect the degradation behavior of the peptides. A higher phosphate concentration in buffer solutions causes higher degradation, however. The ionic strength of buffer solutions has no significant influence on stability. Solutions of gonadorelin and triptorelin, respectively, buffered with acetate (0.1 *M*, pH 5.0) with 3% (w/v) mannitol as an additive show a predicted $t_{90\%}$ of 9.0 years and 7.7 years at 20°C, respectively.

KEY WORDS: gonadorelin; triptorelin; stability; aqueous solution; buffers; pH–rate profile.

INTRODUCTION

Gonadorelin is a decapeptid (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) which was first isolated from porcine hypothalami by Schally *et al.* in 1971 (1). In its analogue triptorelin the Gly^6 is replaced by D-tryptophan (2), which causes higher biological activity by enhancing stability to enzymatic degradation and increasing receptor binding affinity.

The therapeutic aspects and clinical uses of gonadorelin and its analogues were recently reviewed in a number of publications (3–6). Gonadorelin causes a stimulation of the pituitary–gonadal function, while its analogues after a primary stimulation inhibit the gonadal function by receptor down regulation. Many preparations in liquid and freeze-dried forms or recently also as prolonged controlled-release dosage forms are currently available on the market (7,8). Nevertheless, there have been only a few investigations reported about stability of these peptides (9–16).

The purpose of this investigation was to study the pH-dependent stability of gonadorelin and triptorelin in aqueous solution and the influence of several buffer species on the peptides.

MATERIALS AND METHODS

Kinetic Studies

pH–Rate Profiles

Seven buffer solutions, consisting of varying amounts of 0.01 *M* citric acid and 0.02 *M* phosphate solutions, were prepared at each specific pH (pH 2.2, pH 3, pH 4, . . . , pH 8). The pH of all solutions was measured at the appropriate temperature (Model pHm 64, Radiometer, Copenhagen, Denmark). Sample solutions were prepared by dissolving gonadorelin (as diacetate, 99.5% purity, Ferring Arzneimittel, Kiel, Germany) and triptorelin (as acetate, 98.3% purity, Ferring Arzneimittel, Kiel, Germany), respectively, in a fixed volume (10.0 ml) of the above buffer solutions to make approximately 200 μg/ml. The solutions (8 ml) were filled into sterile type I brown glass vials (10 ml, Macherey-Nagel, Düren, Germany) under laminar air flow (Typ ASW Q, Bleymehl, Jülich, Germany) and sterilefiltration (0.2 μm, Schleicher & Schüll, Dassel, Germany), sealed with sterile, Polytef-lined stoppers (Pharma-Gummi, Eschweiler, Germany), and stored in a dark oven (Model U30, Memmert, Schwabach, Germany) maintained at 50 ± 0.2°C for up to 28 days. Samples were taken immediately after preparation and from the oven after 7, 14, 21, and 28 days of storage and immediately placed in a freezer (−20°C) until all samples for the 28 days had been collected. The samples (0.5 ml) were taken from the vials by sterile syringes and needles through the stoppers and filled into micro test tubes (1.5 ml, Eppendorf, Hamburg, Germany). Before analysis, the samples were removed from the freezer, equilibrated to room temperature, and well shaken. The pH value for each sample was checked after analysis to ensure no significant pH change at each designated sampling time compared to initial conditions. The concentration of gonadorelin and triptorelin, respectively, was determined in triplicate by stability-indicating reversed-phase HPLC methods.

Buffer Comparison

Three buffer solutions of varying buffer species (ace-

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tate, 0.1 *M;* phosphate, 1/15 *M;* citric acid/phosphate, 0.1/0.2 *M*) were prepared at pH 5.0. Each buffer solution was also used after 10-fold dilution. Changing of pH was compensated by adding acetic acid, phosphoric acid, and sodium hydroxide, respectively. The samples were prepared, stored, collected, and handled as described above, except storage duration was up to 84 days and samples were taken after 14, 28, 42, 56, 70, and 84 days of storage.

Ionic Strength

Potassium chloride was added to a 0.1 *M* acetate buffer solution, pH 5.0, in suitable amounts to make 0.1, 0.2, and 0.5 *M* solutions related to potassium chloride. Changing of pH was compensated by adding sodium hydroxide. The samples were prepared, stored, collected, and handled as described above (see Buffer Comparison).

Arrhenius Plot

A buffer solution of acetate, 0.1 *M*, with 3% (w/v) mannitol as an additive (12) was prepared at pH 5.0. Samples were prepared, collected, and handled as described above. The samples were stored in dark ovens (Memmert, Schwabach, Germany) maintained at 30, 40, 50, 60, 73, and 80 ± 0.2°C, respectively, for up to 300 days (30°C). The samples were taken in temperature-specific intervals (e.g., 1 day at 80°C and up to 30 days at 30°C).

HPLC Methods

The high-performance liquid chromatograph (HPLC) consisted of a dual-piston pump (Model PU, 4011, Pye Unicam, Cambridge, England) and a variable-wavelength UV absorbance detector (Model PU 4020, Pye Unicam, Cambridge, England). Injections were made with a 20-μl constant-volume injection valve (Rheodyne, Cotati, California). Chromatographic conditions for gonadorelin were as follows: a Vydac C 18 protein and peptide column, 5 μm, 250 × 4.6-mm id (The Separations Group, Hesperia, USA), using a mixture (v/v) of 0.2 *M* triethylammonium phosphate buffer (pH 2.0) and tetrahydrofuran (90:10) as mobile phase at a flow rate of 1 ml/min and UV detection at 210 nm. Chromatographic conditions for triptorelin were as follows: a Shandon ODS Hypersil column, 3 μm, 60 × 4.6-mm id (Shandon Labortechnik, Frankfurt, Germany), using a mixture (v/v) of 0.2 *M* triethylammonium phosphate buffer (pH 2.0) and acetonitrile (78:22) as mobile phase at a flow rate of 1 ml/min and UV detection at 210 nm. The peak areas of the peptides and their degradation products were recorded using a computing integrator (Model PU 4810, Pye Unicam, Cambridge, England) at a speed of 0.5 cm/min. Standard curves were constructed each day for calibration over a range of 100 to 300 μg/ml. A control solution was assayed after each analysis to ensure the reproducibility of the HPLC procedures. The concentration of the peptides was determined by comparing their peak areas to that of external standard solutions. The HPLC methods were validated by checking the selectivity, linearity, sensitivity, and precision of method and system (15). The initial concentration of each drug solution was designated 100%; all subsequent concentrations were expressed as a percentage of the initial concentration.

RESULTS AND DISCUSSION

The correlation coefficient of the detector linearity for gonadorelin and triptorelin at the concentration range of 100 to 300 μg/ml was found to be greater than 0.999. The reproducibility at this concentration range was also calculated and shown to be less than 0.5% SD (n = 6). Figures 1 and 2 illustrate the HPLC chromatograms of gonadorelin and triptorelin stored at pH 8.0 at 50 ± 0.2°C for 28 days. The amounts of the remaining intact decapeptides were 42.4 and 47.1%, respectively. The degradation products were eluated separately without apparent interference with the peaks of the intact peptides. With the exception of the free acid (peak 4 in Figs. 1 and 2), the degradation products have not been identified. The free acid was also shown to be formed in stability investigations of nafarelin, the D-Nal(2)[6] analogue of gonadorelin, in aqueous solution at several pH ranges (10). D-Ser[4]-gonadorelin and D-Ser[4]-triptorelin are likely products at high pH since racemization of Ser[4] was shown to occur in a similar gonadorelin analogue by Nishi *et al.* (14).

The pH-dependent stability of gonadorelin and triptorelin in aqueous solution is similar. The pH–rate profiles are mirrored in Fig. 3. The data points are connected by line segments to illustrate the V-shaped curve of the profiles. The

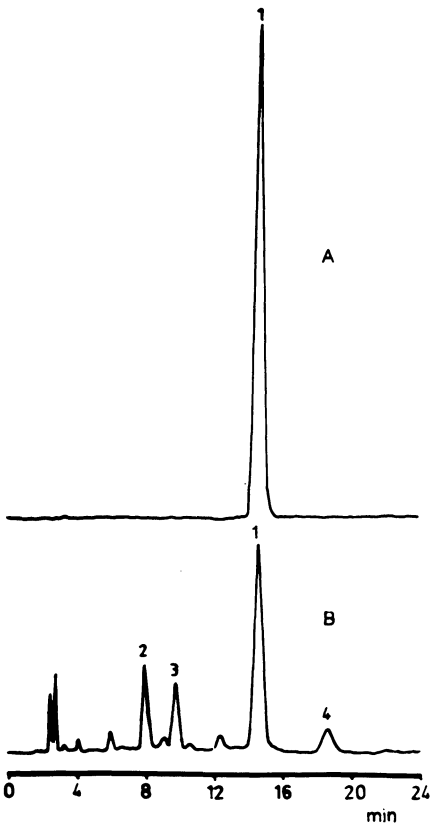

Fig. 1. HPLC recordings of gonadorelin (peak 1) and its main degradation products (peaks 2, 3, and 4) stored at pH 8.0 (0.01/0.02 *M* citric acid/phosphate buffer) at 50 ± 0.2°C for 0 days (A) and 28 days (B).

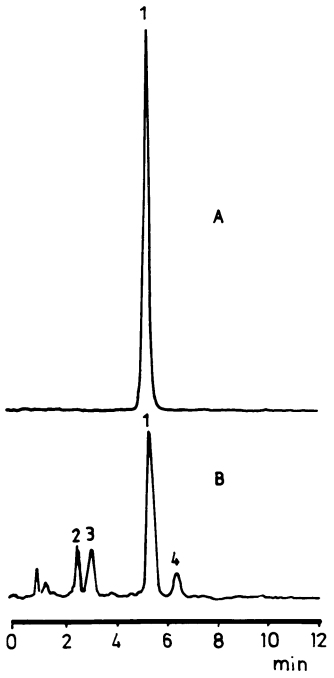

Fig. 2. HPLC recordings of triptorelin (peak 1) and its main degradation products (peak 2, 3, and 4) stored at pH 8.0 (0.01/0.02 *M* citric acid/phosphate buffer) at 50 ± 0.2°C for 0 days (A) and 28 days (B).

results show a maximum stability of gonadorelin and triptorelin, respectively, at a pH of about 5.0.

Data showing the stability of the peptides in aqueous solution (ca. 200 μg/ml) over the pH range of 2.2–8.0 at 50 ± 0.2°C are listed in Table I. The results indicate an overall apparent pseudo-first-order degradation kinetics. The observed rate constants were obtained from the slopes of the semi-log plots of concentration vs time by statistical regression analysis. The correlation coefficient for all pH conditions was greater than 0.97. The buffer capacities were sufficient to maintain constant pH values as evidenced by no observed pH change for all solutions throughout the entire period of study.

The $t_{90\%}$ of gonadorelin and triptorelin, calculated based

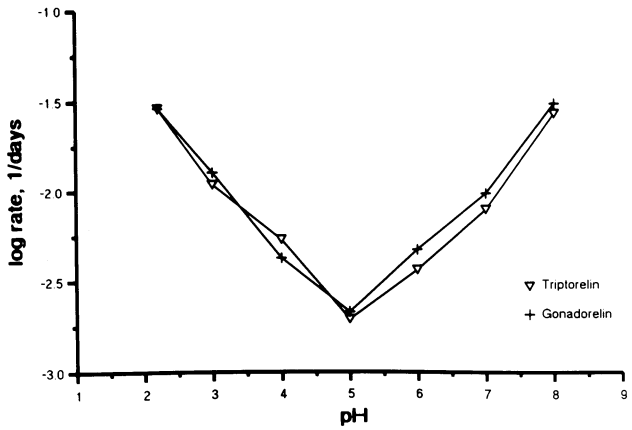

Fig. 3. pH–rate profile of the degradation of gonadorelin and triptorelin, respectively, in 0.01/0.02 *M* citric acid/phosphate buffer, pH 2.2–8.0, at 50 ± 0.2°C for 28 days.

Table I. Rate Constants and $t_{90\%}$ for the Degradation of Gonadorelin and Triptorelin in 0.01/0.02 *M* Citric Acid/Phosphate Buffer, pH 2.2–8.0 at 50 ± 0.2°C

	Gonadorelin		Triptorelin	
pH	Rate constant (10^{-3} days^{-1})	$t_{90\%}$ (days)	Rate constant (10^{-3} days^{-1})	$t_{90\%}$ (days)
2.2	29.31	3.6	29.24	3.6
3.0	12.89	8.1	11.05	9.5
4.0	4.32	24.3	5.49	19.1
5.0	2.17	48.4	1.97	53.3
6.0	4.81	21.8	3.72	28.2
7.0	9.74	10.8	7.93	13.2
8.0	30.44	3.5	26.82	3.9

on the $t_{90\%} = 0.105/(\text{rate})$ equation, are also shown in Table I. Depending on the pH the $t_{90\%}$ ranged from 3.5 to 48.4 days for degradation of gonadorelin at a pH range of 2.2–8.0 at 50 ± 0.2°C. The $t_{90\%}$ of triptorelin ranged under the same conditions from 3.6 to 53.3 days for degradation.

The ionic strength of the buffer solutions has no significant influence on the stability of gonadorelin and triptorelin in aqueous solution. The degradation rates of the peptides in buffer solutions with potassium chloride are not very different from those in pure buffer solution as shown in Table II.

Table III shows the rate constants for the tested buffer species at two concentrations at pH 5.0. The lowest decomposition appears in acetate buffer, while phosphate buffer causes a higher degradation. Thus, acetate has a smaller catalytic effect on degradation of gonadorelin and triptorelin in aqueous solution than phosphate. This might be connected with the higher basicity of phosphate in relation to acetate. There was no remarkable difference between the higher and the lower concentrated acetate buffer as mirrored in Table III. However, in the case of the citric acid/phosphate buffer and, particularly, the phosphate buffer, the higher buffer concentration causes greater degradation, apparently because of a catalytic effect of phosphate. Thus, in addition to pH, the buffer species strongly affects the stability of gonadorelin and triptorelin in aqueous solution. The interpretation of pH–rate profiles based on data observed in experiments with different buffer species (10) should be treated with caution, at least as far as stability investigations of peptides are concerned.

Arrhenius plots of the data observed in kinetic studies at six different temperatures (30–80°C) are reasonably linear,

Table II. Rate Constants and $t_{90\%}$ for the Degradation of Gonadorelin and Triptorelin in 0.1 *M* Acetate Buffer, pH 5.0, with Various Amounts of Potassium Chloride at 50 ± 0.2°C

	Gonadorelin		Triptorelin	
Amount of KCl	Rate constant (10^{-3} days^{-1})	$t_{90\%}$ (days)	Rate constant (10^{-3} days^{-1})	$t_{90\%}$ (days)
0.0 M	1.20	87.5	1.17	89.7
0.1 *M*	1.20	87.5	1.27	82.7
0.2 *M*	1.26	83.3	1.18	89.0
0.5 *M*	1.19	88.2	1.25	84.0

Table III. Rate Constants and $t_{90\%}$ for the Degradation of Gonadorelin and Triptorelin in Various Buffer Solutions at pH 5.0 and 50 ± 0.2°C

Buffer[a]	Gonadorelin		Triptorelin	
	Rate constant (10^{-3} days^{-1})	$t_{90\%}$ (days)	Rate constant (10^{-3} days^{-1})	$t_{90\%}$ (days)
A, 0.01 *M*	1.17	89.7	1.21	86.8
A, 0.1 *M*	1.20	87.5	1.17	89.7
B, 1/150 *M*	1.98	53.0	2.07	50.7
B, 1/15 *M*	3.07	34.2	3.21	32.7
C, 0.01/0.02 *M*	2.04	51.5	2.07	50.7
C, 0.1/0.2 *M*	2.62	40.1	2.63	39.9

[a] Acetate (A), phosphate (B), citric acid/phosphate (C).

with a correlation coefficient greater than 0.99 (Fig. 4). The slopes give an activation energy of the overall reaction of 95.99 kJ mol^{-1} for gonadorelin and 92.04 kJ mol^{-1} for triptorelin. Extrapolation to 20°C gives a predicted mean $t_{90\%}$ at pH 5.0 of 9.0 years, with a minimum $t_{90\%}$ of 5.5 years (95% confidence) for gonadorelin and 7.7 years with a minimum $t_{90\%}$ of 5.1 years (95% confidence) for triptorelin. These findings agree with the described mean $t_{90\%}$ of 3.8 years for nafarelin at 25°C at pH 5.4 in acetate buffer (10).

The results of the stability investigations of gonadorelin and triptorelin in aqueous solution seem to verify the statement of van Nispen, who recommends a slightly acid pH for least decomposition of peptides in solution (18). Those generalizations, however, should be defined carefully, since the stability of peptides in solution strongly depends on the experimental conditions, the composition and pH of the buffer solution, and the composition and primary and secondary structure of the peptides.

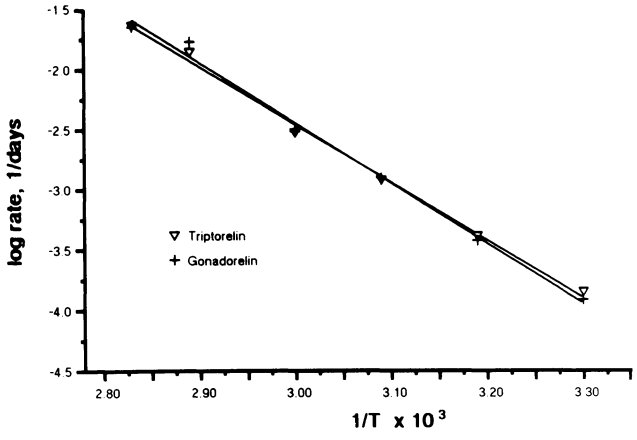

Fig. 4. Arrhenius plot of log rate constant vs 1/*T* for the degradation of gonadorelin and triptorelin, respectively, in 0.1 *M* acetate buffer with 3% (w/v) mannitol at pH 5.0.

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