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Stability of the synthetic pentapeptide thymopentin in aqueous solution: Effect of pH and buffer on degradation

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Summary

The influence of pH and various buffer species (acetate, borate, citrate and phosphate) on the stability of thymopentin in aqueous solution at 50°C has been studied using a stability-indicating high-performance liquid chromatographic method. This analytical procedure shows a linear response range at concentrations of 50–200 μg/ml with a correlation coefficient greater than 0.999. The observed rate of degradation was found to follow apparent first-order kinetics with respect to thymopentin. An exact pH optimum of thymopentin in aqueous solution cannot be defined. The peptide shows a similar stability at pH ranges of approximately 5.5–8.0. These stability plateaus can be observed in all buffer systems. The influence of the various buffer species was shown to be different. Acetate has the most favourable effect on stability while phosphate causes greater degradation.

Introduction

Thymopentin (Ortho Pharmaceutical Corp., Raritan, U.S.A.; 96% purity by area, HPLC) is the synthetic pentapeptide Arg-Lys-Asp-Val-Tyr that corresponds to amino acid residues 32–36 of the thymic hormone thymopoietin. First synthesized in 1979, thymopentin (TP-5) was shown to be the minimal fragment of its parent molecule that reproduces the biological activities of thymopoietin (Goldstein et al., 1979).

It is pleiotropic in action, affecting neuromuscular transmission, induction of early T-cell differentiation and immune regulation (Goldstein and Audhya, 1985). Thymopentin has a short half-life of approx. 30 s in human plasma caused by enzymatic degradation (Tischio et al., 1979). There are some further investigations dealing with the stability of the pentapeptide in plasma (Audhya and Goldstein, 1983, 1985; Heavner et al., 1986), but nothing has been reported about the stability in aqueous solution.

The purposes of this investigation were to study the pH-dependent stability of thymopentin in aqueous solution and to clarify the influence of several buffer species on the peptide.

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Materials and Methods

Kinetic studies

19 buffer solutions of four different buffer systems (Table 1) were prepared at each specific pH. The pH of all solutions was measured at the

TABLE 1

Composition and sources of the tested buffer systems

Buffer system	pH range	Component I	Component II	Source
Citric acid/phosphate	2.2–8.0	citric acid, 0.1 M	Na_2HPO_4, 0.2 M	(Geigy, 1968)
Borate/hydrochloric acid	7.7–9.2	HCl, 0.1 M	$Na_2B_4O_7$, 0.2 M	(Geigy, 1968)
Acetate	4.5–6.5	CH_3COOH, 0.1/0.01 M	$NaCH_3COO$, 0.1/0.01 M	(Dolder and Skinner, 1975)
Phosphate	5.0–8.0	NaH_2PO_4, 1/15 M	Na_2HPO_4, 1/15 M	(Geigy, 1968)

appropiate temperature (Model pHm 64, Radiometer, Copenhagen, Denmark).

Sample solutions were prepared by dissolving thymopentin (as acetate hydrate) in a fixed volume (10.0 ml) of the above buffer solutions to make approx. 100 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 sterile filtration (0.2 μm, Schleicher and 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 42 days. Samples were taken immediately after preparation and from the oven after 7, 14, 21, 28, 35 and 42 days of storage and immediately placed in a freezer (−20°C) until all samples for the 42 days had been collected. In the case of citric acid/phosphate and borate buffer, samples were only taken up to 35 days. 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 thymopentin was determined in triplicate by a stability-indicating reversed-phase HPLC method.

HPLC method

The high-performance liquid chromatograph (HPLC) consisted of a dual piston pump (Model PU 4011, Pye Unicam, Cambridge, U.K.) and a variable-wavelength UV absorbance detector (Model PU 4020, Pye Unicam). Injections were made with a 20 μl constant volume injection valve (Rheodyne, Cotati, U.S.A.). Chromatographic conditions for thymopentin were as follows: a μ-Bondapak C 18 column, 10 μm, 250 × 3.9 mm i.d. (Waters Millipore, Eschborn, Germany), using a mixture (v/v) of 0.02 M potassium dihydrogen phosphate buffer (pH 3.0) and methanol (90:10) as mobile phase at a flow rate of 3 ml/min and UV detection at 210 nm. The peak area of the peptide and those of its degradation products were recorded using a computing integrator (Model PU 4810, Pye Unicam) at a speed of 0.5 cm/min. Standard curves were constructed each day for calibration over a range of 50–200 μg/ml. A control solution was essayed after each analysis to ensure the reproducibility of the HPLC procedures. The concentration of the peptide was determined by comparing its peak area to those of external standard solutions. The HPLC method was validated by checking the selectivity, linearity, sensitivity and precision of method and system (Debesis et al., 1982). The initial concentration of each drug solution was designated as 100%; all subsequent concentrations were expressed as a percentage of the initial concentration.

Results and Discussion

The correlation coefficient of the detector linearity for thymopentin at the concentration range of 50–200 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% S.D. ($n = 6$). Fig. 1 illustrates the HPLC chromatogram of thymopentin stored at pH 2.2 at

$50 \pm 0.2°C$ for 35 days. The amount of the remaining intact pentapeptide was 38.5%. The degradation products were eluted separately without apparent interference with the peaks of the intact peptides. The degradation products have not been identified. Because of the easy cleavage of the peptide bonds of the Asp residue in dilute acid (Schultz, 1967), the corresponding di- and tripeptides can be regarded as degradation products. As described for the degradation of secretin, a heptacosapeptide with two Asp residues, in aqueous solution (Beyerman et al., 1981), the occurrence of the α-β-transpeptidation of the Asp residue in thymopentin is also possible. The exact degradation mechanism of thymopentin in aqueous solution, however, and its degradation products need to be studied further.

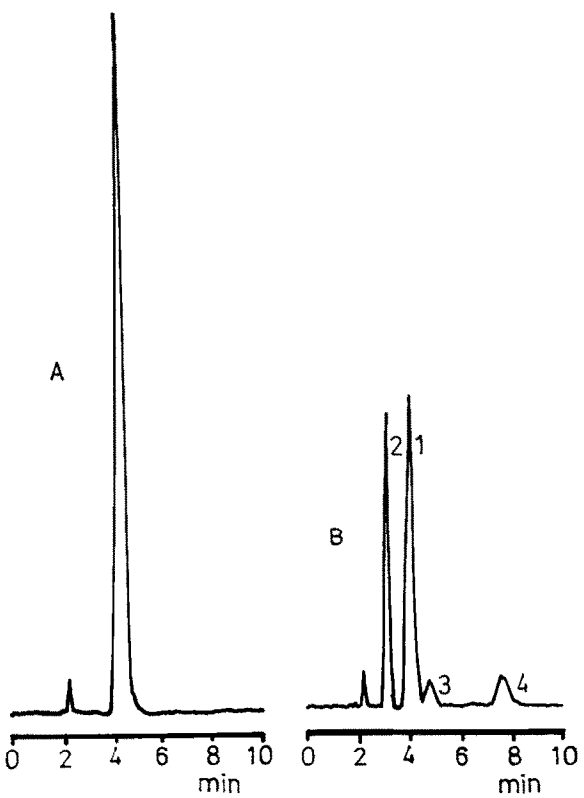

Fig. 1. HPLC recordings of thymopentin (peak 1) and its main degradation products (peaks 2–4) stored at pH 2.2 (0.1/0.2 M citric acid/phosphate buffer) at $50 \pm 0.2°C$ for 0 days (A) and 35 days (B).

TABLE 2

Rate constants and $t_{1/2}$ for the degradation of thymopentin in 0.1/0.2 M citric acid/phosphate buffer pH 2.2–pH 8.0 at $50 \pm 0.2°C$

pH	Rate constant (10^{-3} day^{-1})	$t_{1/2}$ (day)
2.2	26.92	25.7
3.0	25.47	27.2
4.0	20.57	33.7
5.0	13.22	52.4
6.0	5.35	129.5
7.0	5.87	118.1
8.0	3.93	176.3

Data showing the stability of thymopentin in aqueous solution (~ 100 μg/ml) over the pH range of 2.2–8.0 at $50 \pm 0.2°C$ are listed in Table 2. The results indicate an overall apparent pseudo-first order degradation kinetics. Fig. 2 shows ideally the degradation profile of thymopentin in 0.1/0.2 M citric acid/phosphate buffer solution pH 2.2 at $50 \pm 0.2°C$ for 35 days.

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 0.98. The buffer capacities were sufficient to maintain constant pH values as demonstrated by no observed pH change for all solutions throughout the entire period of study.

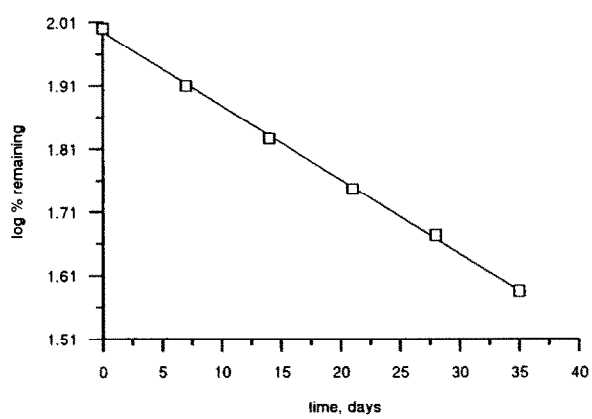

Fig. 2. First-order plot for the degradation of thymopentin in 0.1/0.2 M citric acid/phosphate buffer at pH 2.2 and $50 \pm 0.2°C$.

TABLE 3

Rate constants and $t_{1/2}$ for the degradation of thymopentin in various buffer species and different pH ranges at 50 ± 0.2°C

Buffer System	pH	Rate constant (10^{-3} day^{-1})	$t_{1/2}$ (day)
Borate	7.7	2.28	247.5
	8.4	2.51	276.1
	9.2	3.93	176.3
Phosphate	5.0	2.88	240.6
	6.0	2.33	297.4
	7.0	2.74	252.9
	8.0	2.76	251.1
Acetate	4.5	5.87	118.1
	5.0	3.37	205.6
	5.5	1.94	357.2
	6.0	1.69	410.1
	6.5	1.87	370.6

The estimated half-life of thymopentin calculated based on the half-life = 0.693/(rate) equation is also shown in Table 2. Depending on the pH the half-life ranged from 25.7 days to 176.3 days at a pH range of 2.2–8.0 at 50 ± 0.2°C. To verify the degradation behavior of thymopentin in aqueous solution at pH ranges > 8.0 a borate/hydrochloric acid was used. Table 3 lists the degradation rates and half-lives of thymopentin in borate buffer pH 7.7, 8.4 and 9.2 at 50 ± 0.2°C for 35 days. The degradation profiles of the peptide under these conditions are shown in Fig. 3.

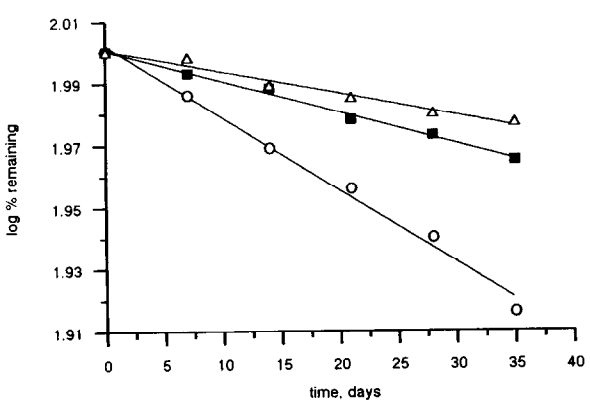

Fig. 3. First-order plots for the degradation of thymopentin in borate/hydrochloric acid buffer at pH 7.7 (△), pH 8.4 (■) and pH 9.2 (○) and 50 ± 0.2°C.

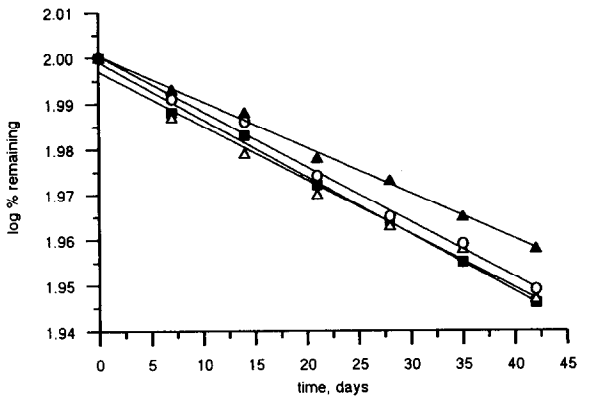

Fig. 4. First-order plots for the degradation of thymopentin in phosphate buffer at pH 5.0 (■), pH 6.0 (▲), pH 7.0 (△) and pH 8.0 (○) and 50 ± 0.2°C.

The comparison of the slopes by a statistical test (Sachs, 1969) results in an insignificant difference between the slopes of the degradation profiles at pH 7.7 and 8.4 ($\alpha = 5\%$). The highest degradation, however, occurs in the buffer solution of pH 9.2.

Data showing the stability of thymopentin solutions (100 μg/ml), buffered with phosphate and acetate, respectively, over the pH range of 5.0–8.0 and 4.5–6.5, respectively at 50 ± 0.2°C for 42 days are also listed in Table 3.

The degradation profiles of thymopentin in phosphate and acetate buffer solution are shown in Figs 4 and 5, respectively. The highest stability

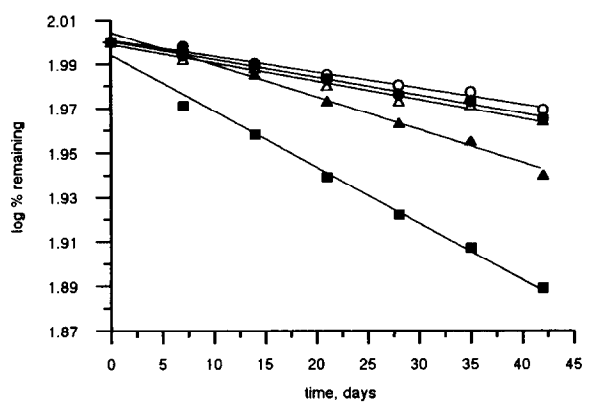

Fig. 5. First-order plots for the degradation of thymopentin in acetate buffer at pH 4.5 (■), pH 5.0 (▲), pH 5.5 (△), pH 6.0 (○) and pH 6.5 (●) and 50 ± 0.2°C.

in phosphate buffer is detectable at pH 6.0. The degradation rates at pH 5.0, pH 7.0 and pH 8.0 are not significantly different ($\alpha = 5\%$).

The differences between the slopes of the degradation profiles in acetate buffer at pH 5.5, 6.0 and 6.5 are not significant ($\alpha = 5\%$), while pH ranges < 5.5 cause higher degradation.

The pH-rate profiles of thymopentin in the four different buffer systems are shown in Fig. 6. An exact pH optimum of thymopentin in aqueous solution cannot be defined. The peptide shows a similar stability at pH ranges of approx. 5.5–8.0. These stability plateaus can be observed in all buffer systems. The plateaus are, however, located on different levels.

The degradation profiles of thymopentin in three different buffers, citric acid/phosphate, acetate and phosphate, are compared in Fig. 7. The slopes of all profiles are significantly different ($\alpha = 1\%$). The maximum stability of the pentapeptide was shown to be in acetate buffer. Phosphate ions in the solution cause greater degradation. The concentration and ionic strength of the buffer systems were not mentioned, since it was shown in investigations with two decapeptides that these factors do not affect the stability of peptides in aqueous solution (Helm and Müller, 1990). Acetate buffer should be used in further stability testings of thymopentin. The positive influence of acetate buffer on peptides in solution was also described by Van Nispen (1987). Moreover, the

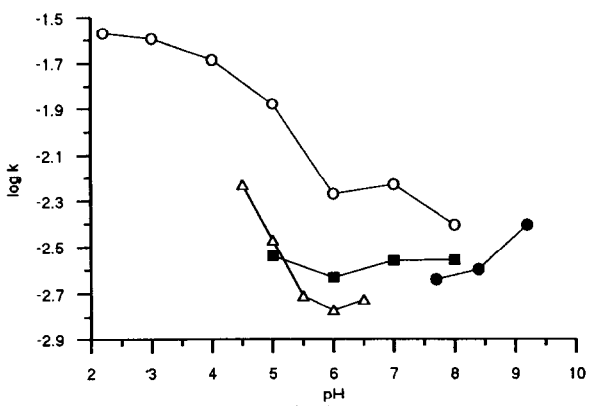

Fig. 6. pH rate profiles of the degradation of thymopentin in various buffer species at 50±0.2°C; citrate buffer (○), borate buffer (●), acetate buffer (△) and phosphate buffer (■).

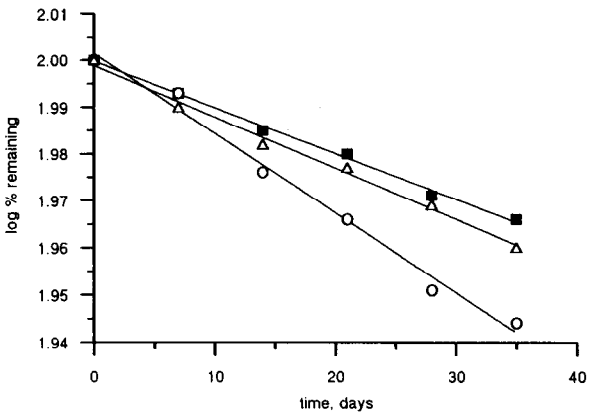

Fig. 7. First-order plots for the degradation of thymopentin in acetate buffer (■), phosphate buffer (△) and citric acid/phosphate buffer (○) at pH 6.0 and 50±0.2°C.

author recommends a slightly acid pH for the minimum decomposition of peptides in solution (Van Nispen, 1987). However, this is not true for all peptides. Besides the findings of this investigation, a maximum stability at neutral pH ranges is also reported for the heptacosapeptide secretin (Tsuda et al., 1990).

Therefore, generalizations 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 also on the composition and primary and secondary structure of the peptides.

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