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Kinetics of degradation of a cyclic lactam analog of α-melanotropin (MT-II) in aqueous solution

Sydney O. Ugwu [a], En-Ling Lan [a], Shubh Sharma [b], Victor Hruby [b] and James Blanchard [a]

[a] *Department of Pharmaceutical Sciences, College of Pharmacy, University of Arizona, Tucson, AZ 85721 (USA)* and [b] *Department of Chemistry, University of Arizona, Tucson, AZ 85721 (USA)*

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Summary

The kinetics of degradation of MT-II in aqueous buffered solution was studied in order to facilitate the formulation of a stable oral dosage form. A stability-indicating high-performance liquid chromatographic (HPLC) assay was used to measure the concentrations of MT-II remaining at various time periods. The rate of degradation of MT-II was studied as a function of pH, phosphate buffer concentration, temperature and ionic strength. Results indicated that the degradation of MT-II followed apparent first-order kinetics. The pH-rate profile showed that MT-II was most stable at approximately pH 5.0. Data obtained from this study also indicated that the degradation rate of this peptide was directly proportional to phosphate buffer concentration and temperature. The shelf-life of MT-II in aqueous buffer solutions at 25°C was 27 h. The activation energy was 7.5 kcal/mol. The degradation rate of MT-II appeared to be independent of the ionic strength of the aqueous buffered solution.

Introduction

MT-II is a cyclic heptapeptide derivative of α-melanocyte stimulating hormone (Fig. 1) (Al-Obeidi et al., 1989). MT-II stimulates melanin synthesis and thereby tans the skin rapidly (Hadley et. al., 1989). This peptide is currently in phase I clinical trials for use in the prevention of sunlight-induced skin cancers (Griego and Levine, 1992).

The potential therapeutic importance of this peptide prompted us to undertake a study of its chemical stability and degradation kinetics in aqueous buffered solutions, as no previous data were available. Furthermore, stability data obtained from this study will provide fundamental information needed to develop stable delivery systems of MT-II for both animal and human studies.

The purpose of this investigation was to determine the influence of pH, phosphate buffer concentration, temperature and ionic strength on the rate of degradation of MT-II.

Correspondence to: J. Blanchard, Department of Pharmaceutical Sciences, College of Pharmacy, University of Arizona, Tucson, AZ 85721, U.S.A.

Materials and Methods

Materials

Purified MT-II was obtained from Dr Victor Hruby of the Department of Chemistry, University of Arizona. The purity of the peptide was greater than 99% as determined by reversed-phase HPLC on a C_{18} bonded silica column, with UV detection at 280 and 220 nm. The MT-II sample was a powdered lyophilized diacetate salt form. HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Potassium phosphate, monobasic and dibasic (analytical grade), was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). The water used in the experiments was deionized and distilled using a Millipore filter system (Millipore Corp., Bedford, MA, U.S.A.).

HPLC analysis

The HPLC system consisted of a Spectra-Physics (Fremont, CA, U.S.A.) Isochrom pump, a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector with a 50 μl loop and a Hitachi/Spectra-Physics (Fremont, CA, U.S.A.) Model 100-30 variable-wavelength UV detector set at 214 nm. The analytical column was a Zorbax (Dupont Instruments, Wilmington, DE, U.S.A.) C_8 column (10 μm, 150 mm $\times$ 4.6 mm i.d.), fitted with a Whatman (Clifton, NJ, U.S.A.) C_{18} (30 μm) guard column (10 mm $\times$ 4.6 mm i.d.). Peak recording and area integrations were made with a Spectra-Physics model 4400 integrator. The mobile phase consisted of 0.1 M aqueous K_2HPO_4:acetonitrile (73:27% v/v) containing 18 μl 99% v/v triethylamine per l of mobile phase (pH 2.5). A flow rate of 1.0 ml/min was utilized. Duplicate 100 μl injections were made for each sample assayed using a Hamilton (Reno, NV, U.S.A.) Model 702-SNR 100 μl syringe.

Kinetic studies

A stock solution of MT-II (1000 μg/ml) was prepared in water and stored at 4°C until use. This solution was prepared fresh weekly. The sample solution (10 μg/ml) was prepared by mixing appropriate volumes of MT-II stock solution, phosphate buffer (0.5 M) and potassium chloride (1.5 M). Potassium chloride (1.5 M) and KOH or HCl (1.0 M) were used to adjust ionic strength and pH of the solution, respectively. In some experiments, it was necessary to use a low phosphate buffer concentration (0.02 M), to minimize buffer catalysis and a low ionic strength (μ) to reduce any possible salt effect. The temperature dependence of MT-II degradation was studied at 50, 60, and 70°C in 0.02 M phosphate buffer (pH 7.0, μ = 0.15). The influence of different μ (0.15, 0.25, 0.5, 0.75, 1.25, 1.5) was studied at 60°C (0.02 M phosphate, pH 9.11). The degradation of MT-II in the pH range 2–9 was studied at 60°C, with μ and phosphate buffer concentration held constant at 0.15 and 0.02 M, respectively. The effect of different phosphate buffer concentrations (0.02, 0.10, 0.5M) was investigated at 60°C (μ = 1.5, pH 9.11). The pH of all sample

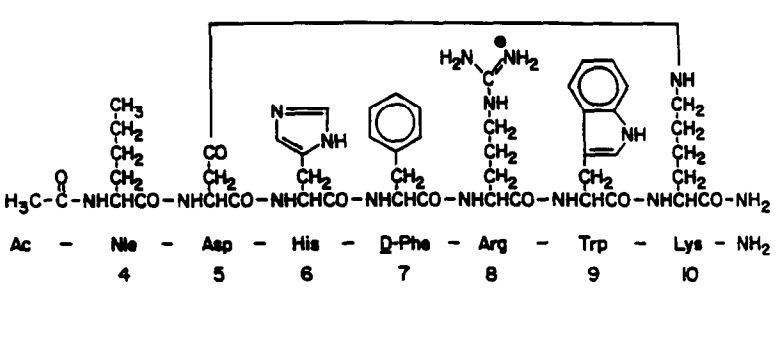

Fig. 1. Structure of α-melanotropin analog (MT-II).

solutions was measured at the various study temperatures. All sample solutions were placed in 5-ml screw-capped polypropylene tubes and stored in an oven set at the desired temperature. At appropriate time intervals, aliquots of samples were withdrawn and analyzed by HPLC.

Results and Discussion

HPLC analysis

The assay was found to be linear between 1 and 10 μg/ml MT-II ($r = 0.9997$). The assay precision of a 2 μg/ml MT-II sample was less than 1% RSD ($n = 5$). The homogeneity and identity of the MT-II peak were determined as reported elsewhere (Ugwu and Blanchard, 1992). The stability-indicating nature of this assay is shown by the chromatograms (Fig. 2) of samples of MT-II (10 μg/ml) in pH 2.4 and 7.0 buffer solutions that were stored at 4°C for 300 days. The amount of the intact MT-II remaining was 33%. The degradation peaks eluted separately without any interference from the peak representing the intact peptide. The degradation mechanism and the identification of the major degradation products have not yet been determined.

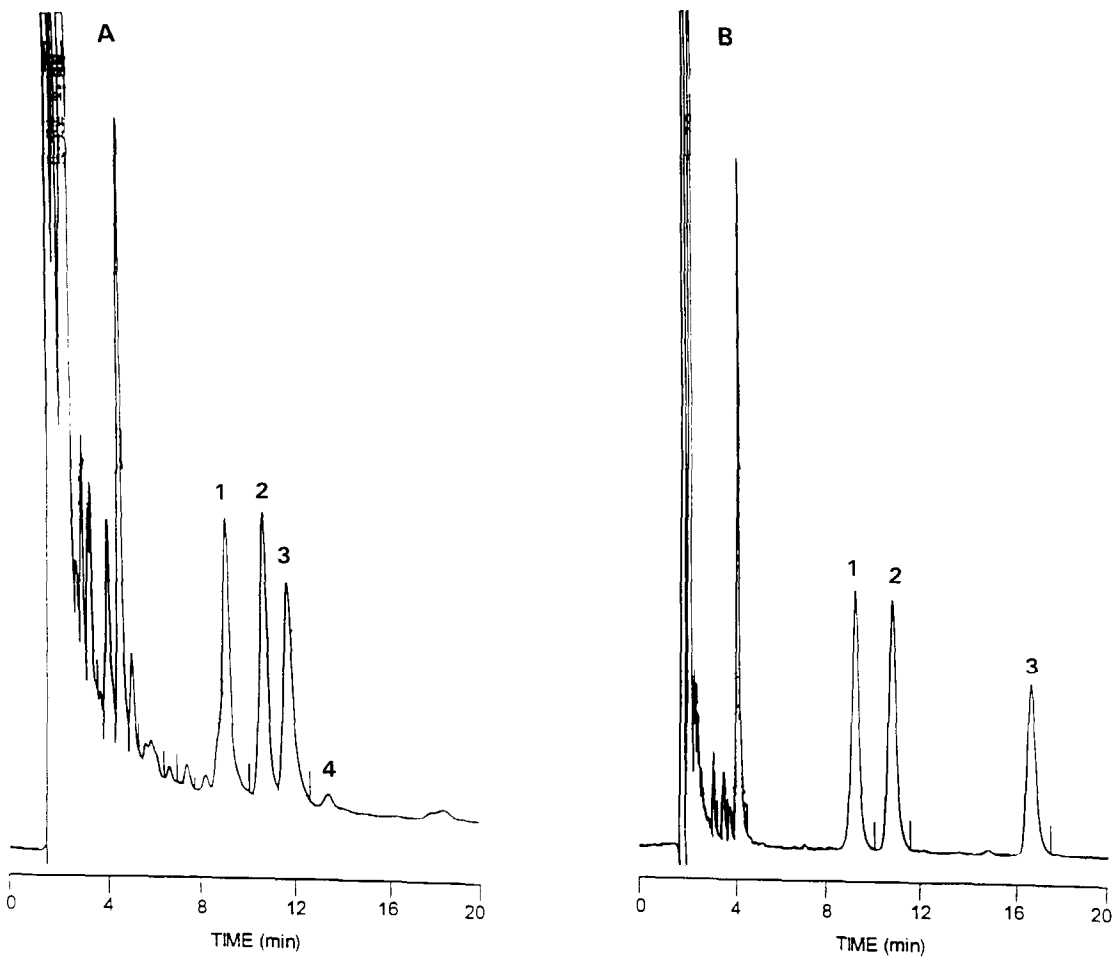

Fig. 2. Chromatograms of MT-II (10 μg/ml) in (A) pH 2.4 and (B) pH 7.0, 0.02 M phosphate buffers when stored at 4°C for 300 days. (1) MT-II, (2–4) degradation products. The numbers assigned to the degradation products in A and B are arbitrary and are not meant to imply that the peak so numbered represents identical molecules.

MT-II degradation kinetics

A typical logarithmic plot of the percentage of MT-II remaining vs time for the degradation kinetics in pH 7.0, 0.02 M phosphate buffer is shown in Fig. 3. The plot indicates that the degradation of MT-II in the aqueous buffer solution followed apparent first-order kinetics under conditions of constant pH, temperature and μ. The apparent first-order rate constant (K_{obs}) was obtained from the slope of the following equation:

$$\ln \%A_t = -K_{obs}t + \ln \%A_0$$

where $\%A_0$ and $\%A_t$ denote the percentage of MT-II remaining at time zero and time t, respectively, and K_{obs} represents the degradation rate constant.

The effect of temperature on degradation rate

The effect of temperature on the degradation rate of MT-II was investigated at 50, 60 and 70°C in 0.02 M phosphate buffer at pH 7.0 and μ = 0.15. At all three temperatures, decomposition of MT-II followed an apparent first-order process. The values of K_{obs} calculated at the different temperatures are summarized in Table 1. The rate constant (K_{obs}) of many chemical reactions can be empirically related to the absolute temperature (T) by the Arrhenius equation:

$$\log K_{obs} = \log A - E_a/2.303RT$$

TABLE 1

Observed rate constants, Arrhenius parameters, and shelf-lives for MT-II degradation [a,b,c]

Temperature (°C)	K_{obs} (h^{-1})	t_{90} (h)	$\Delta H^{\dagger}$ (kcal/mol)
50 ± 0.2	0.0102	10.29	6.89
60 ± 0.2	0.0155	6.77	6.87
70 ± 0.2	0.0202	5.87	6.85

[a] Conditions: pH 7.0, μ = 0.15 and [phosphate] = 0.02 M.
[b] Activation energy (E_a) = 7.5 kcal/mol.
[c] Log $K_{obs} = -E_a/2.303RT + \log A$.

where A, E_a and R are the pre-exponential constant, experimental activation energy and gas constant, respectively. In this study, the dependence of K_{obs} on temperature followed a linear Arrhenius plot (Fig. 4). The linear Arrhenius plot obtained here indicates that the degradation mechanism did not change over the temperature range studied. On the basis of these data, the E_a calculated from the slope of the Arrhenius plot was 7.53 kcal/mol. The pre-exponential constant was calculated from the intercept to be 1301.4 h^{-1}. The enthalpy ($\Delta H^{\dagger}$) at the different temperatures (Table 1) was calculated as follows:

$$\Delta H^{\dagger} = E_a - RT$$

and the shelf-life at 25°C was calculated to be 26.92 h from the equation:

$$t_{90} = 0.105/K_{obs}$$

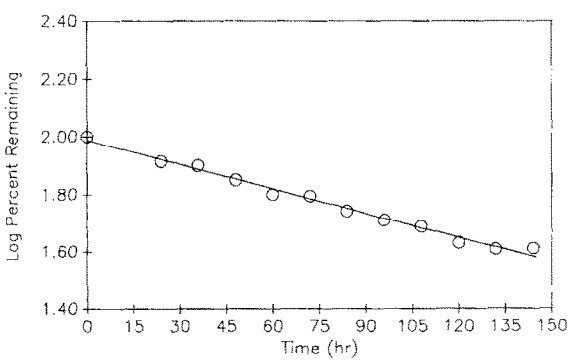

Fig. 3. Typical apparent first-order plot for the degradation of MT-II in pH 7.0, 0.02 M phosphate buffer (μ = 0.15) at 60 ± 0.2°C.

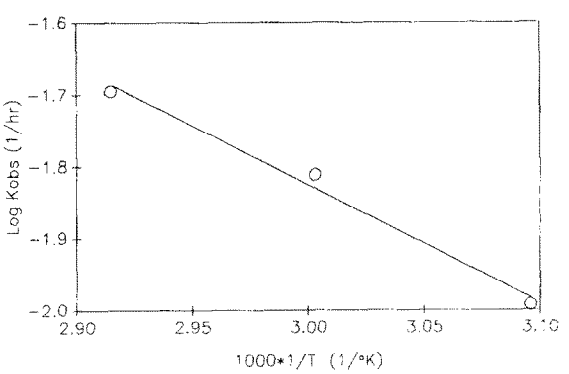

Fig. 4. Arrhenius plot of the degradation of MT-II in pH 7.0, 0.02 M phosphate buffer and constant μ of 0.15 (r^2 = 0.98).

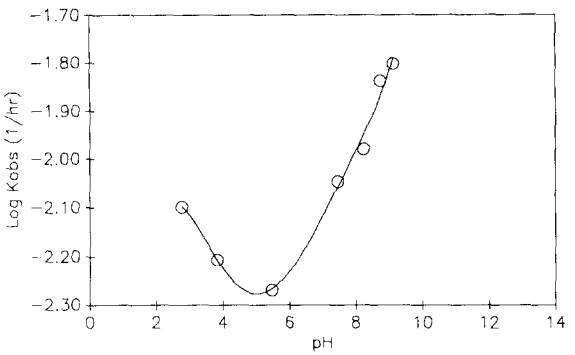

Fig. 5. pH-rate profile for the degradation of MT-II in 0.02 M phosphate buffer, pH 2.0–9.5, $\mu = 0.15$, at 60 ± 0.2°C.

where t_{90} represents the time at which 10% degradation has occurred.

The above shelf-life calculation was made on the assumption that the E_a remained constant over the temperature range studied.

pH-rate profile

The rate of degradation of MT-II at various pH values followed apparent first-order kinetics over the time course of the studies. From the pH-rate profile, shown in Fig. 5, the pH of optimum stability was estimated to be approx. 5.0. A general hypothetical rate equation for this process can be written as follows:

$$K_{obs} = K_{H^+}[H^+]^n + K_o + K_{OH^-}[OH^-]^m$$

where K_{H^+}, K_{OH^-} and K_o are the rate constants for the specific acid-, base- and water-catalyzed reactions, respectively. The decreasing (n) and increasing (m) slopes of the pH-rate profile were estimated to be -0.102 and 0.127, respectively. Therefore, the orders with respect to hydrogen and hydroxide ions were -0.102 and 0.127, respectively. The other three parameters (K_{H^+}, K_{OH^-} and K_o) were obtained by fitting the pH-rate data to above equation using a nonlinear least-square regression program. The estimated n and m values were fixed during the regression analysis. The best fit of the data provided values of $K_{H^+} = 0.015$ (S.E. = 0.004), $K_o = -0.003$ (S.E. = 0.002), and $K_{OH^-} = 0.047$ (S.E. = 0.006). Since K_o is not significantly different from zero the rate equation can be written as follows:

$$K_{obs} = 0.015[H^+]^{-0.102} + 0.047[OH^-]^{0.127}$$

From the above analysis, the following conclusions can be made:

(1) The degradation reaction is not specifically acid- or base-catalyzed by hydrogen or hydroxide ion, respectively, since the slopes in the acid or base regions of the pH-rate profile were neither $+1$ nor -1, respectively. This finding may indicate that MT-II degrades by several different pathways, each with its own pH dependence and true catalytic coefficient.

(2) The uncatalytic term (water) does not play a role in the degradation of MT-II.

(3) The hydroxyl ion catalyzes MT-II degradation to a greater degree than the hydrogen ion.

Effect of ionic strength

The following modified form of the Debye-Huckel equation was used to examine the effect of ionic stregth on the degradation rate of MT-II:

$$\log K_{obs} = \log K_o + 2QZ_AZ_B\left[\sqrt{\mu}\,/\left(1 + \sqrt{\mu}\right)\right]$$

where K_o is the rate constant at $\mu = 0$, Q denotes a constant for a given solvent and temperature, and Z_A and Z_B are the charges on species A and B, respectively. In this study, the plot of log K_{obs} against $\sqrt{\mu}\,/(1 + \sqrt{\mu})$ gave a slope that

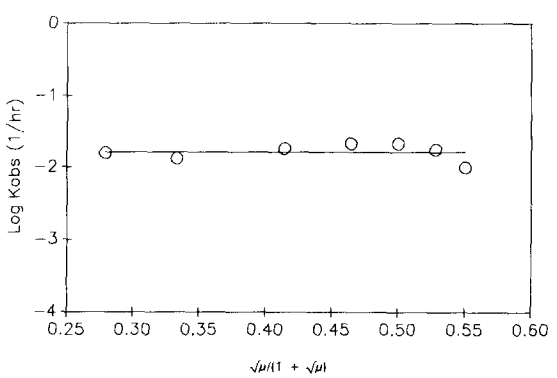

Fig. 6. Effect of ionic strength on the degradation of MT-II in 0.02 M phosphate buffer, pH 9.11 at 60 ± 0.2°C.

was not significantly different from zero ($p = 0.983$) (Fig. 6). Thus, the kinetic salt effect on the degradation kinetics of MT-II was interpreted to be negligible.

Effect of phosphate buffer concentration

An increase in phosphate buffer concentration increases the rate of degradation of MT-II, as shown in Fig. 7. MT-II diacetate may exist as the neutral, monoacidic or diacidic cationic species depending on the pH of the reaction medium. Therefore, in aqueous media, the following expressions may be written for MT-II:

$$BH_2^{2+} \rightleftharpoons BH^+ + H^+ \quad (1)$$

$$BH^+ \rightleftharpoons B + H^+ \quad (2)$$

where B, BH^+ and BH_2^{2+} denote the neutral, monoacidic and diacidic cationic species, respectively. The effect of buffer concentration on the rate of degradation was studied at a constant pH of 9.11. At this pH, the equilibrium represented by Eqn 2 predominates, since pH $\gg pK_1$. Therefore, it can be assumed that either BH^+ or B can undergo degradation. The following kinetic schemes may then be written:

$$BH^+ \rightarrow \text{products} \quad (3)$$

or

$$B \rightarrow \text{products} \quad (4)$$

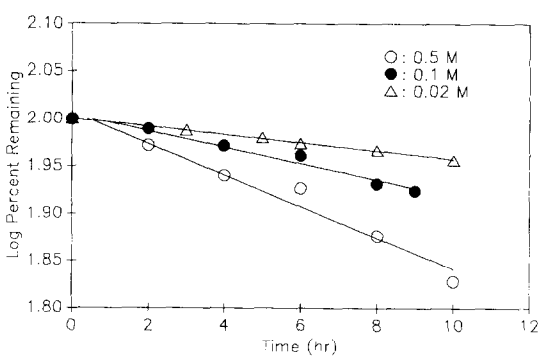

Fig. 7. Effect of phosphate buffer concentration on the degradation of MT-II, pH 9.11 and $\mu = 1.5$, at 60 ± 0.2°C.

In the above reactions, the two forms of MT-II can react with hydroxide ion, hydrogen ion, or the phosphate buffer species to form products. In a previous experiment, we had shown that the ionic strength had no effect on the rate of degradation of MT-II. Therefore, the possibility of two charged species reacting can be eliminated from consideration since one of the reactants must be the neutral species (Carstensen, 1970). Since hydroxide ion, hydrogen ion, and the phosphate buffer species are all charged, it follows that the uncharged reactant has to be the neutral form (B) of MT-II. Therefore, the reaction represented by Eqn 3 can be assumed not to occur. From Eqn 4, the following generalized rate equation may be written as follows:

$$d(\text{products})/dt = K_{obs}[B] \quad (5)$$

K_{obs} can be decomposed into individual equations for specific acid/base and general acid/base catalysis. Hence, the generalized rate equation may be written as:

$$K_{obs} = K_o + K_1[H^+] + K_2[OH^-] + K_3[H_2PO_4^-] + K_4[HPO_4^{2-}] \quad (6)$$

Since the rate of degradation was observed to increase with an increase in phosphate buffer concentration, the contribution of general acid/base catalysis to the overall rate of degradation is highly significant. At the experimental pH (9.11), the predominant phosphate buffer species is HPO_4^{2-} and the hydrogen ion concentration is very low. Therefore, the overall rate equation can be simplified to:

$$K_{obs} = K_2[OH^-] + K_o + K_4[HPO_4^{2-}] \quad (7)$$

Summary and Conclusions

From the above results, it may be concluded that MT-II is relatively stable and has the potential to be successfully formulated into an oral solid (tablet or capsule) dosage form, or a liquid

dosage form to be taken soon after preparation. However, the pH of all formulations should be maintained as close to 5.0 at as low a phosphate buffer concentration as possible. Finally, such aqueous formulations should be stored at refrigerator temperature (4°C) unless used within 24 h of preparation.

The effects of temperature, ionic strength and phosphate concentration on MT-II degradation were not studied at the pH of optimum stability in order to accelerate the degradation process and hence obtain data more quickly. Nevertheless, these results should closely mimic the pattern observed at pH 5.0.

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