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Partitioning properties and degradation kinetics of the [Nle^4-DPhe^7]α-MSH analog Melanotan-I (MT-I)

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Abstract

The objectives of this research were to determine the partitioning properties and the solution stability of the [Nle^4-DPhe^7]α-MSH analog called Melanotan-I (MT-I). The apparent partition coefficients (APC) were determined in two solvent systems, i.e. octanol-buffer and isooctane-buffer in order to calculate Δ Log P.C. (partition coefficient) values. The Δ Log P.C. values at pH 2.5 and 7.4 were -0.307 and -0.409, respectively. The solution stability of MT-I was determined by accelerated stability studies at 70°, 80° and 87°C at pH 7.4 and ionic strength (μ) of 0.17. The energy of activation (E_a) was calculated to be 15.83 kcal/mol, resulting in an estimated shelf life of 40 days at room temperature. From the pH-rate profile, MT-I was observed to be relatively stable under acidic conditions and exhibited greater degradation under basic conditions. Both ionic strength (μ = 0.15 to 1.5, at 0.05 M buffer concentration) and phosphate buffer concentration (0.1–0.5 M, μ = 1.5) at pH 7.3 and 80°C had no effect on the degradation kinetics of MT-I.

Keywords: Melanotan-I; Partition coefficients; Degradation kinetics

1. Introduction

Melanotan-I (Fig. 1) is a tridecapeptide (mol. wt. 1647), an analog of α-MSH (Sawyer et al., 1980). It is currently in Phase I trials at the Arizona Cancer Center (University of Arizona, Tucson) to evaluate its potential as a chemopreventive agent for sunlight-induced skin cancers. We have previously determined the transport properties and the bioavailability of MT-I in the Caco-2 cell monolayer model and a rat in-situ model, respectively (Surendran et al., 1995a). In order to formulate suitable delivery systems for this peptide for use in both animals and humans, it was necessary to obtain information on certain physicochemical properties of MT-I for example, partition coefficients (P.C.s), aqueous solution stability, etc.

Although P.C.s determined in octanol-buffer systems have been correlated with absorption potential for conventional drug molecules, for peptide-based drugs that are capable of hydrogen

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bonding, Δ Log P.C., i.e. Log ($P.C._{\text{hydrogen bonding solvent}}/P.C._{\text{non-hydrogen bonding solvent}}$) has been observed to be a more accurate predictor of the absorption potential (Burton et al., 1992). In this study, octanol and isooctane were chosen as the hydrogen bonding and non-hydrogen bonding solvents, respectively.

In order to estimate the shelf life of this peptide, accelerated stability studies were conducted at 70°, 80°, and 87°C (pH 7.4, μ = 0.17) and the various kinetic parameters were determined by fitting the data to the Arrhenius equation. The effect of pH, ionic strength (μ) and buffer concentration on MT-I stability was also determined. MT-I was quantitated using a specific, sensitive and stability-indicating HPLC assay recently developed in our laboratory (Surendran et al., 1995b).

2. Materials and methods

2.1. Materials

Purified MT-I ($\geq$ 99% pure) was obtained from Bachem (Torrance, CA). HPLC-grade acetonitrile was from Burdick and Jackson (Muskegon, MI). Radioactive MT-I (^{3}H) was a generous gift from Dr. M.E. Hadley, Department of Anatomy, University of Arizona. Ready Caps^R were purchased from Beckman Instruments (Fullerton, CA). Potassium phosphate (monobasic and dibasic) and potassium chloride were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Purified HPLC grade octanol and isooctane were purchased from Sigma (St. Louis, MO). The water used in the experiments was deionized and distilled using a Millipore filter system (Millipore Corp., Bedford, MA).

2.2. HPLC analysis

The HPLC system consisted of a Spectra-Physics (Fremont, CA) Isochrom pump, a Rheodyne (Cotati, CA) Model 9125 injector valve with 40 μl loop and a Spectra-Physics Model 100 variable-wavelength UV detector set at 214 nm. The analytical column was a Vydac (Hesperia, CA) C_8 (5 μm) microbore cartridge (150 mm $\times$ 2.1 mm I.D.) fitted with a Whatman (Clifton, NJ) C_{18} (10 μm) guard column (10 mm $\times$ 4.6 mm I.D.). The guard column was routinely changed after about 100 injections as a precautionary measure to avoid pressure build-up in the HPLC system. Peak recording and integrations were made with a Spectra-Physics Model 4290 integrator. All injections were made with a Hamilton (Reno, NV) Model 725-SNR syringe previously coated with Sigmacote^R (Sigma, St. Louis, MO) to minimize binding of MT-I to syringe components. The mobile phase consisted of 0.1 M K_2HPO_4: acetonitrile (80:20%, v/v) with 18 μl/l of 99% (v/v) triethylamine at pH 2.50 at a flow rate of 1 ml/min. Fresh stock solutions of MT-I (1 mg/ml) in water were prepared weekly and stored at -20°C until use.

2.3. Partition coefficients

The apparent partition coefficients (APC) were determined in two solvent systems (i.e. *n*-octanol-buffer and isooctane-buffer) at two different pH values, 2.5 and 7.4. Phosphate buffer (0.05 M, μ = 0.15) was prepared at the appropriate pH and this solution was used to saturate the organic solvents by vigorous stirring at room temperature for 90 min. This mixture was transferred to a separatory funnel and the two layers allowed to separate for at least 24 h. On the day of an experiment, appropriate volumes of phosphate buffer pre-saturated with the organic solvent and a fresh stock solution of MT-I (1 mg/ml) were mixed in 15-ml glass tubes sealed with teflon-lined screw caps (Baxter Scientific Products Division, McGaw Park, IL) previously coated with Sigmacote^R to eliminate non-specific binding of MT-I to the glass walls. Radioactive MT-I (specific activity 25 Ci/mmol) was added to each tube. A 50-μl aliquot of this solution was directly transferred to Ready Caps^R to determine the initial concentration of MT-I. The organic solvent was then layered onto the MT-I solution at an aqueous/organic volume ratio of 1:10. The tubes were closed tightly, transferred to a rotator and allowed to equilibrate for 48 h. The organic solvent was removed by aspiration, the aqueous

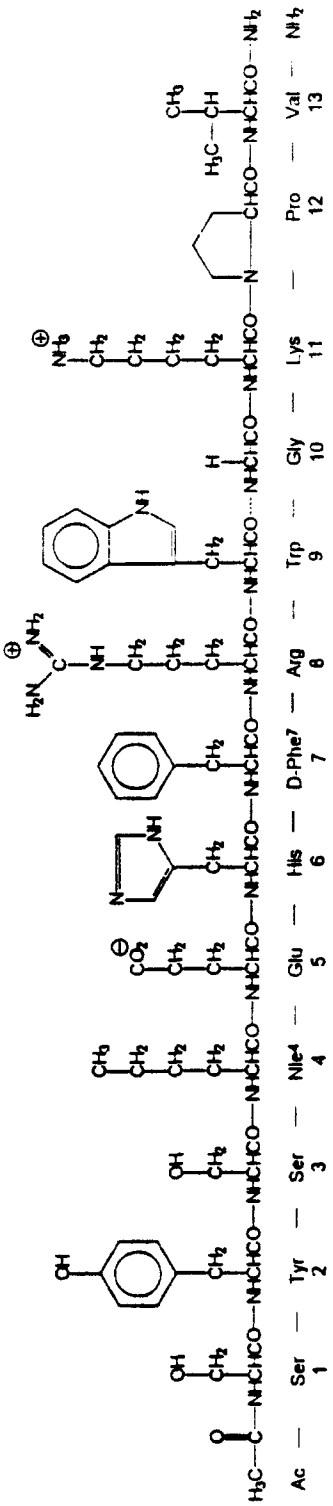

Fig. 1. Structure of [Nle^4-DPhe^7]α-MSH (Melanotan-I).

layer carefully transferred to silanized Eppendorf tubes (Sigma, St. Louis, MO) and centrifuged for 10 min. Fifty microliters of the resulting supernatant were transferred to Ready Caps^R. The Ready Caps^R were then dried at 25°C for 10–12 h and counted using a scintillation counter (Beckman LS 5000TD) in order to quantitate MT-I.

In order to verify the partition coefficient values obtained, the experimental protocol was repeated using an independent equilibration and phase-separation method, i.e. the use of the Mixxor apparatus (Lidex Technologies Inc., Bedford, MA) according to our protocol described previously (Lan et al., 1994). The aqueous phases before and after equilibration were then assayed for MT-I content using our HPLC method (Surendran et al., 1995b). APCs were determined using the following equation after adjusting for the difference in the phase volume ratio:

$$\text{APC} = \frac{(\text{Initial conc.} - \text{Final conc.})_{\text{aqueous}}}{(\text{Final conc.})_{\text{aqueous}}}$$

$$\Delta \log \text{P.C.} = \log (\text{APC}_{\text{octanol}}/\text{APC}_{\text{isooctane}})$$

2.4. Kinetic studies

Sample solutions of MT-I (20 μg/ml) were prepared by mixing appropriate volumes of MT-I stock solution (1 mg/ml) in phosphate buffer. Potassium chloride (1.5 M) was used to adjust the ionic strength and KOH or HCl (1.0 M) was used to adjust the pH of the solutions. The temperature dependence of MT-I degradation was studied at 70°, 80°, and 87°C in 0.05 M phosphate buffer (pH 7.4, μ = 0.17). The degradation of MT-I in the pH range 2.5–11.04 was studied at 80°C with μ and phosphate buffer concentration held constant at 0.17 and 0.05 M, respectively. The influence of different μ (0.15, 0.25, 0.50, 0.75, 1.00, 1.25, 1.5) was studied at 80°C in 0.05 M phosphate buffer, pH 7.3. The effect of different phosphate buffer concentrations (0.1, 0.25 and 0.5 M) was investigated at 80°C at μ of 1.5 and pH 7.3. The pH of all sample solutions was determined at the various study temperatures. All sample solutions were placed in 10-ml screw-capped teflon-lined glass vials (Baxter Scientific Products Division, McGaw Park, IL) previously coated with Sigmacote^R and stored in an oven set at the desired temperature. At appropriate intervals, aliquots of samples were withdrawn and analyzed by HPLC.

3. Results and discussion

MT-I is a superpotent analog of α-MSH. When administered to laboratory animals and humans, it causes skin darkening (tanning). Although this peptide is under Phase I clinical trials the University of Arizona Cancer Center, our study is the first report on its partitioning properties and solution stability kinetics.

3.1. MT-I partition coefficients

The results of the partitioning studies are summarized in Table 1. While the octanol-buffer partition coefficient data are generally considered to be a predictor of the absorption potential of a compound, it appears that such relationships often may not be very meaningful for peptides and proteins. For peptide-based drugs, consideration of the possible hydrogen-bonding of the polar groups of the peptide and therefore the desolvation energy required to transfer the peptide molecule into the cell membrane also appears to be important (Burton et al., 1991). This desolvation energy can be determined experimentally by measuring by Δ Log P.C. using a hydrogen-bond-

Table 1
Summary of partitioning properties of MT-I

pH	Octanol-buffer[a]	Isooctane-buffer[a]	Δ Log P.C.[b]
2.5	0.0221 ± 0.003[c]	0.0448 ± 0.006[c]	−0.3068 ± 0.05[c]
7.4	0.0196 ± 0.003	0.0496 ± 0.002	−0.4093 ± 0.09

[a]Apparent partition coefficients, Mean ± S.D., n = 4, [phosphate] = 0.05 M, μ = 0.15.
[b]Δ Log P.C. = Log (P.C.$_{\text{octanol}}$/P.C.$_{\text{isooctane}}$).
[c]No significant differences detected at $P < 0.05$ between this value and the value obtained at pH 7.4.

ing and a non-hydrogen bonding solvent (Burton et al., 1991). From our studies, it was observed that MT-I had a relatively low octanol-buffer APC, both at pH 2.5 and 7.4. However, an examination of the Δ Log P.C. data indicates that the desolvation energy required to transfer MT-I from the aqueous membrane interface into the hydrophobic interior of the membrane is relatively small at these pH values. For example, it has been reported that di-peptides and tri-peptides with molecular weights ranging from 514 to 556 have Δ Log P.C. values in the range of 4–7 (Burton et al., 1992). Thus, while the low octanol-buffer APC suggests that partitioning of MT-I into the polar head group of the phospholipid bilayer may be a major limiting factor controlling the membrane permeability of MT-I, the relatively low Δ Log P.C. is likely to facilitate an overall increase in its membrane transport. Since the Δ Log P.C. has been suggested to be the principal factor determining the transport of peptides (Burton et al., 1992), the absorption potential of MT-I can be predicted to be fairly good. In fact, studies in our laboratory (Surendran et al., 1995a) with in-vitro (Caco-2) and in-situ (rat) models have shown that both transport (in the Caco-2 model) and bioavailability (in the in-situ rat model) of MT-I was much greater than one might predict from its octanol-buffer partition coefficient alone. However, in the above study it was also established that degradation of MT-I by proteases associated with the absorptive cells was one of the major obstacles to its delivery and that inhibition of this degradation by co-administration of protease inhibitors like aprotinin was a promising approach. Thus, although Δ Log P.C. appears to be a more accurate predictor of membrane permeability than the octanol-buffer partition coefficient, for peptide and protein drugs, it seems prudent to evaluate their transport in a suitable in-vitro or in-situ system in order to determine the contribution of other unfavorable processes (e.g. degradation by proteases) that may serve as the overriding and rate-limiting determinant of the successful oral delivery of these agents.

One interesting observation is the lack of an effect of pH on the partitioning behavior of MT-I. MT-I has five ionizable groups, i.e. Glu and Tyr which are acidic with theoretical pK_a values of 4.3 and 10.9, respectively, and His, Lys and Arg which are basic with theoretical pK_a values of 6.0, 10.8 and 12.5, respectively. Therefore, at pH 2.5, MT-I will carry a net charge of $+3$, and at pH 7.4, a net charge of $+1$. Based on this information, one would expect the partitioning of MT-I at pH 7.4 to be higher than at pH 2.5. In fact, a similar relationship between pH (and therefore the net charge on the molecule) and APC was demonstrated previously in our laboratory for another α-MSH analog known as Melanotan-II (Lan et al., 1994). Nevertheless, our data indicate that there is no significant difference ($P > 0.05$) in the partition coefficients and Δ Log P.C. at these two pH values. One explanation for this observation could be the existence of ion-pairs between the positive charges on MT-I and the negative ions of the phosphate buffer, thereby rendering the molecule temporarily neutral and thus modulating the effect of pH on partitioning into the organic phase. This possibility seems reasonable since the concentration of MT-I used was 5.47×10^{-7} M whereas the buffer concentration was 0.05 M. However, further studies need to be conducted to validate this 'ion-pairing' hypothesis. Finally, degradation of MT-I within the time frame of the study (48 h) was eliminated as a confounding factor since the t_{90} (the time for 10% degradation to occur) for pH 2.5 and 7.3 at room temperature (25°C) is 60 days and 40 days, respectively. APCs were not determined at alkaline pH since appreciable degradation was noted at pH values greater than 8.0. For example, the t_{90} at room temperature and pH 8.7 is 117 h.

3.2. MT-I degradation kinetics

The stability-indicating nature of the HPLC assay is shown in Fig. 2. The temperature dependence of MT-I degradation is summarized in

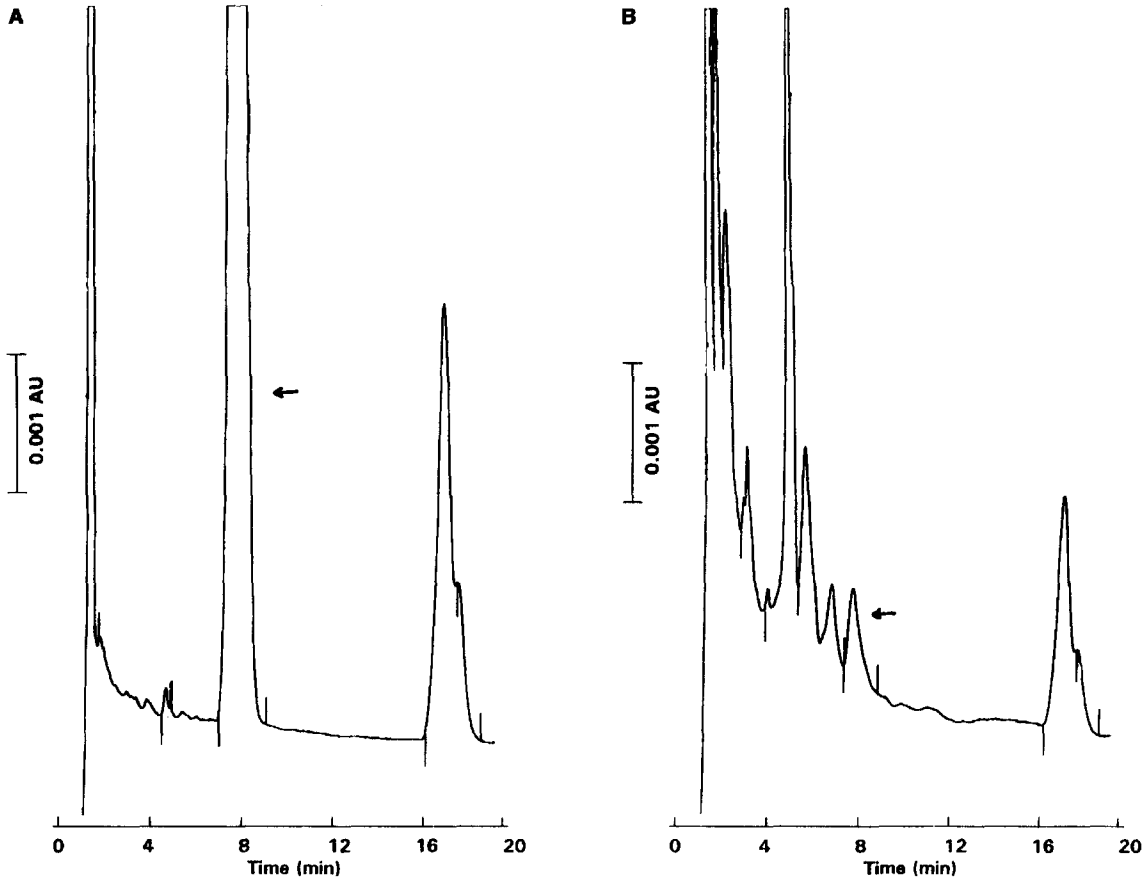

Fig. 2. Chromatograms demonstrating the stability-indicating nature of the HPLC assay. A 20 μg/ml MT-I solution in 0.1 M phosphate buffer, pH = 7.3, and μ = 1.5 M at (A) t = 0 h (i.e. initial sample) and (B) t = 288 h at 80°C (4.5 half-lives). The arrow on the chromatograms refer to the elution position of MT-I.

Table 2, and Fig. 3. MT-I exhibited apparent first-order kinetics with an energy of activation (E_a) of 15.83 kcal/mol. On the assumption that the E_a remained constant over the temperature range, the rate of degradation at room temperature (25°C) was calculated to be 1.09×10^{-4}/h. Using the equation $t_{90} = 0.1054/K_{obs}$, the shelf life at room temperature was calculated to be 40 days.

Table 2
Temperature dependence of MT-I degradation kinetics[a,b,c]

Temperature (°C)	$K_{obs} \times 10^3$ (h^{-1})	t_{90} (h)	ΔH (kcal/mol)
70 ± 0.2	3.412	30.77	15.14
80 ± 0.2	7.609	13.79	15.12
87 ± 0.2	10.014	10.48	15.11

[a]Conditions: pH 7.4, μ = 0.17 and [phosphate] = 0.05 M.
[b]Activation energy (E_a) = 15.83 kcal/mol.
[c]Log $K_{obs} = -E_a/2.303RT + \log A$.

3.2.1. pH-rate profile

A general rate equation for the pH-dependent degradative process (Fig. 4) can be written as follows:

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

where K_o and K_{OH}^{-} are the rate constants for the specific water-catalyzed and hydroxyl-ion catalyzed reactions, respectively, and m represents the

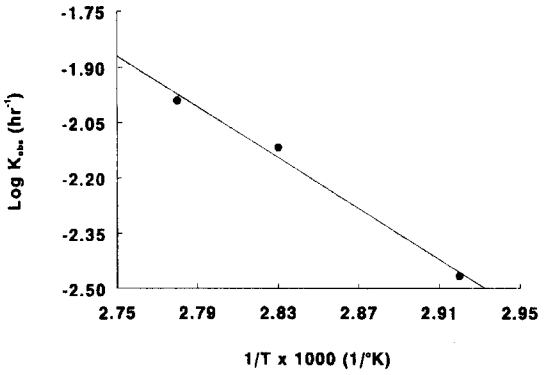

Fig. 3. Arrhenius plot of the degradation of MT-I in pH 7.4, 0.05 M phosphate buffer and μ of 0.17 (r = 0.995).

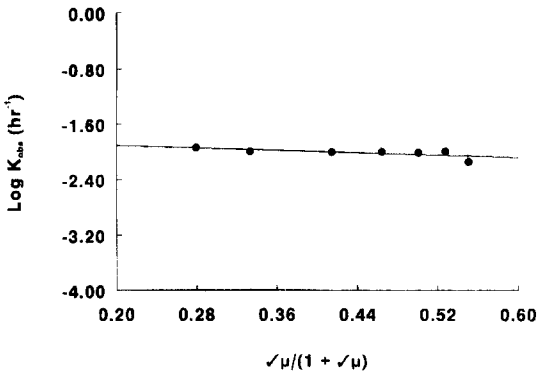

Fig. 5. Effect of ionic strength on the degradation of MT-I in 0.05 M phosphate buffer, pH 7.3 at 80 ± 0.2°C.

positive slope of the base-catalyzed (OH^-) reaction. K_o, K_{OH}^-, and m were obtained by fitting the pH-rate data to the above equation using a non-linear least-squares regression program. The best fit of the data provided values of K_o = −0.002 (S.E. = 0.005), K_{OH}^- = 0.936 (S.E. = 0.087) and m = 0.298 (S.E. = 0.023). Since K_o is not significantly different from zero, the rate equation can be written as follows:

$$K_{obs} = 0.936[OH^-]^{0.298}$$

3.2.2. Effect of ionic strength

The following modified form of the Debye-Hückel equation was used to examine the effect of ionic strength on the degradation rate of MT-I:

$$\log K_{obs} = \log K_o + 2QZ_1Z_2[\sqrt{\mu}/(1+\sqrt{\mu})]$$

where K_o is the rate constant at μ = 0, Q denotes a constant for a given solvent and temperature and Z_1 and Z_2 are the charges on species 1 and 2, respectively. A plot of Log K_{obs} vs. $\sqrt{\mu}/(1+\sqrt{\mu})$ gave a slope that was not significantly different ($P > 0.05$) from zero (Fig. 5). Thus, any possible kinetic salt effect on the degradation kinetics of MT-I was interpreted to be negligible.

3.2.3. Effect of phosphate buffer concentration

The effect of phosphate buffer concentration on the stability of MT-I is shown in Fig. 6. Phosphate buffer concentrations up to 0.5 M did not appear to influence the degradation of this peptide. MT-I has five ionizable groups, two acidic

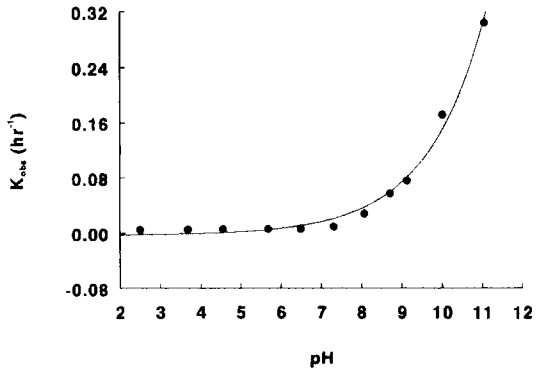

Fig. 4. pH-rate profile for the degradation of MT-I in 0.05 M phosphate buffer, pH 2.5–11.04, μ = 0.17 at 80 ± 0.2°C.

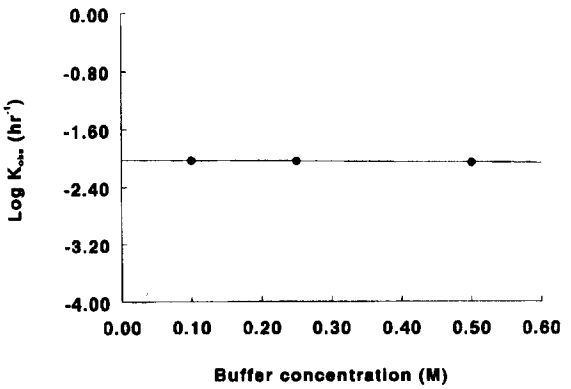

Fig. 6. Effect of phosphate buffer concentration on the degradation of MT-I, pH 7.3 and μ = 1.5, at 80 ± 0.2°C.

and three basic. At pH 7.3, the following expressions can be written for MT-I.

$$A^-BH_2^{2+} \rightleftarrows A^-BH^+ + H^+ \quad (1)$$

$$A^-BH^+ \rightleftarrows A^-B + H^+ \quad (2)$$

Thus, either A^-BH^+ or A^-B can be assumed to undergo degradation. The following kinetic schemes may then be written:

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

or

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

However, it was shown that ionic strength had no effect on the degradation of MT-I. Despite the fact that the large separation of charge in the peptide would weaken the influence of ionic strength, the possibility of two charged species reacting is unlikely since one of the reactants must be a neutral species, i.e. if $Z_1Z_2 = 0$, the net charge on 1 or 2 has to be zero (Carstensen, 1970). Since hydroxide ion, hydrogen ion and the phosphate buffer species are all charged, it is likely that the uncharged reactant is a neutral form of MT-I. An examination of Eqs. (1) and (2) reveals that the only form of MT-I that has a net charge of zero is A^-BH^+. Thus, the following generalized rate equation may be written for the degradation of MT-I.

$$d(\text{products})/dt = K_{obs}[A^-BH^+] \quad (5)$$

From the above analysis, the following conclusions can be made. (1) The degradation reaction of MT-I is not specifically base-catalyzed by the hydroxide ion since the slope of the pH-rate profile in the basic region was not +1. (2) Since the buffer concentration had no effect on the degradation of MT-I, the contribution of general acid/base catalysis to the overall rate of degradation is negligible. (3) The hydrogen ion and the uncatalytic term (water) do not appear to play a significant role in the degradation of MT-I. The possibility that MT-I may degrade by several different pathways, each with its own pH dependence and true catalytic coefficient cannot be ruled out. For example, it is possible that neutral water molecules may be reacting with a deprotonated form of the peptide.

4. Summary and conclusions

From the results of the stability studies, it may be concluded that MT-I is relatively stable and has the potential to be successfully formulated into solid and liquid dosage forms. However, the pH of all formulations should be maintained between 2.5 and 7.4 and the formulations refrigerated to obtain longer shelf lives. Since ionic strength does not appear to influence the degradation of MT-I, electrolytes such as NaCl added to parenteral formulations for the adjustment of osmolality will not be likely to accelerate its degradation. In addition, MT-I is suited for formulations requiring adjustment of pH with phosphate buffer up to concentrations of 0.5 M. However, a study investigating the effect of other commonly used buffer systems, (e.g. citrate, acetate, borate, carbonate) on the degradation kinetics of MT-I is warranted.

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