Isothermal and Nonisothermal Decomposition of Thymopentin and Its Analogs in Aqueous Solution

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Purpose. The degradation kinetics of thymopentin (RKDVY) and its analogs (RKDVW and RPDVY) in aqueous solution was studied by isothermal and nonisothermal methods.

Methods. The isothermal decomposition of thymopentin and its analogs was investigated as a function of pH (2–10), temperature (37, 57, and 80°C) and ionic strength (μ = 0.02 to 1). Nonisothermal decomposition studies were performed using a linear temperature programmer. The temperature increasing rate was set to 0.25°C per hour and the temperature interval varied from 40 to 88°C.

Results. The decomposition of thymopentin and its analogs followed first order kinetics. The dependence of the rate constant on temperature followed a linear Arrhenius plot. This indicated that the degradation mechanism of thymopentin and its analogs might be the same within the temperature range studied. The energies of activation were found to be in close agreement for the isothermal and nonisothermal studies, suggesting that the nonisothermal studies may save considerable amount of time in the early stages of drug development. The logK-pH profile of thymopentin suggests that maximum stability is achieved in the pH range of 6–8.

Conclusions. These results indicate that the nonisothermal methodology provides an attractive alternative to isothermal methods, as it requires a much lower amount of both material and time, to determine the peptide stability and to estimate the shelf-life for peptide pharmaceutical preparations.

KEY WORDS: stability; isothermal; nonisothermal; thymopentin.

INTRODUCTION

Investigation of the decomposition of pharmaceuticals at various temperature to determine Arrhenius parameters and to calculate the shelf-life of pharmaceuticals is the most routinely used method to estimate chemical stability of pharmaceuticals. It is sometimes undesirable, especially at the early development stage, due to the limited materials, analytical resources, and aggressive timelines. The development of a faster, more cost-effective method to evaluate chemical stability would be greatly beneficial. Non-isothermal kinetic methods offer this opportunity. The attractive advantage of non-isothermal kinetics over the traditional isothermal method is that not only can it minimize the amount of material needed, but it also can reduce the total number of experiments and the experimental running time. These are particularly important for peptide pharmaceuticals that they are relatively expensive and are in limited supply to conduct preformulation and formulation screening studies at early development stage. The temperature in the non-isothermal kinetic study is being increased at a predetermined rate. Therefore, from a single experiment, the activation energy, pre-exponential factor, degradation rate constant and the reaction order can be determined.

The non-isothermal method was first introduced when Audibert applied it to study the thermal decomposition of coal (1). Later Baur and Bridges used the linear temperature rise to study the oxidation of metals (2,3). Rogers first applied this non-isothermal method in the pharmaceutical field (4). In the pharmaceutical field, work has predominantly been carried out in solution, except for one study on solid state (5) and one on suspensions (6). However, estimation of the chemical stability of peptide pharmaceuticals using non-isothermal method has not yet been evaluated. So far, the derivative and integral methods are two possible directions in non-isothermal data treatment. Kipp presented a comparison of both data analysis methods (7). Kipp *et al.* also built an automated liquid chromatography workstation to facilitate the data collection in non-isothermal kinetic studies (8,9). Various temperature profiles have been applied in non-isothermal methods. The temperature profiles such as logarithmic, reciprocal, and hyperbolic heating schemes were mainly designed with the goal of simplifying the mathematics involved (4,10,11,12). Unfortunately, the current lack of available hardware to provide the desired temperature-time programs increases experimental difficulty.

Thymopentin, a pentapeptide, which constitutes the segment 32–36 of thymopoietin hormone (a well-defined thymic hormone containing 49 amino acids) is clinically used in the treatment of auto immune diseases, the adjuvant therapy to hepatitis B vaccinations and rheumatoid arthritis (13–16). The potential therapeutic importance of thymopentin led to its being chosen here, along with its analogs, as the model peptides for a greater understanding of peptide drugs stability in aqueous buffered solutions (17). Furthermore, studying the chemical stability and degradation kinetics will provide the fundamental information needed to develop more stable delivery systems of thymopentin and its analogs for animal and human clinical studies.

MATERIALS AND METHODS

Materials

All L-amino acids, Fmoc-amino acids and HMP-Hydroxymethylphenoxymethyl resin were purchased from Perkin Elmer (Foster City, CA). Trifluoroacetic acid (HPLC grade) was purchased from Sigma Chemical Company (St. Louis, MO). HPLC grade acetonitrile was supplied by Fisher Chemical (Fair Lawn, NJ). Distilled-deionized water was used for the preparation of all the solutions. All the other chemicals were analytical grade and were used as received from the commercial suppliers.

Peptide Synthesis, Purification and Characterization

RKDVY, RPDVY and RKDVW pentapeptides were synthesized in the Peptide Synthesis Facility at The University of Texas at Austin using a solid phase peptide synthesizer (Model 430A, Perkin Elmer, Foster City, CA). All the peptides were purified by reversed-phase HPLC using a C18 wide-pore column (DYNAMAX® −300A; 250 × 21.4 mm, 12 μm resin)

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(Rainin, Inc., Woburn, MA). 15 nmole/ml of peptide was vapor phase hydrolyzed using 6N HCl, and both primary and secondary amino acids were derivatized to the phenylthiocarbamyl (PTC) derivative to perform amino acid analysis (Model 421, Perkin Elmer, Foster City, CA). The composition of the purified peptides were characterized by the Protein Sequence Center at The University of Texas at Austin.

HPLC Analysis

The HPLC system consists of a Shimazu UV-VIS detector (SPD-6AV), a solvent delivery pump (LC-600), an integrator (C-R3A) and an auto injector (SIL-9A). A reverse phase C18 (250 × 4.6 mm) column, packed with 5 μm octadecylsilane (Beckman, Palo Alto, CA), was used for HPLC assay at room temperature. Isocratic elution methods consisting of water and acetonitrile in a fixed ratio were developed for peptide analysis. The combination of mobile phases (percent acetonitrile to percent water) and the retention time for thymopentin was 13% v/v and 5.8 mins, for RKDVW pentapeptide was 20% v/v and 5.5 min, and for RPDVY pentapeptide was 16% v/v and 5.1 min. In order to improve peak separation, 0.1% v/v trifluoroacetic acid was added to the mobile phases. The flow rate was maintained at 1.0 ml/min. The detection wavelength was set at 266 nm.

Analytical Method Validation

The HPLC method was validated by injecting six replicates of each standard into the column for three consecutive days. Five standard concentrations were used viz. 7.5, 15, 30, 45, and 60 μg/ml. The assay was found to be linear with r value of 0.999. The coefficient of variation (C.V.%) of the six peak heights ranged from 0.1–0.6% which fell within the minimum and maximum allowable relative standard deviation (RSD) for system precision. The percent difference for theoretical concentrations of 7.5, 15, 30, 45, and 60 μg/ml ranged from −3.88 to 2.04%. The percent recovery values found fall within the acceptable range. The percent coefficient of variation between days for different concentrations was found to be less than 6.1%.

Stability Studies

Buffers

Citrate, sodium phosphate, potassium phosphate and borate buffers were chosen as buffer systems in this study. Citrate buffer system was prepared for the pH range 2 to 8. Borate buffer was prepared for the alkaline pH range 9 to 10. Both sodium phosphate and potassium phosphate buffers were prepared for a pH 7.4 buffer solution. The ionic strength of each buffer was adjusted by adding a calculated amount of potassium chloride. The prepared buffers were stored in a 5°C refrigerator. Prior to use, the temperature of each buffer was equilibrated to room temperature.

Isothermal Study

The oven (Model 5800, Hewlett Packard, Atlanta, GA) temperature was pre-set and maintained at the desired temperatures of 37, 57, or 80°C for isothermal studies. Buffers were pre-equilibrated to the study temperature in the oven for twenty minutes before use. The peptide stock solution (2 mg/ml) was mixed with the pre-heated buffer solutions to yield a final bulk concentration of 40 μg/ml. Aliquots of 300 μl of this bulk solution were placed in 500 μl eppendorf plastic vials; these vials were then tightly capped and stored in the oven at a fixed temperature (37, 57 or 80°C). Vials were removed from the oven at various time intervals and then frozen at −20°C until the HPLC assay was performed. Most of the degradation reactions were carried out for three or more half-lives. The pH of the peptide solutions was confirmed after each sampling step; no significant changes in pH were observed. A volume of 20 μl from each sample was injected into the HPLC system.

Non-Isothermal Study

For non-isothermal studies, samples were prepared same as isothermal study, the oven temperature was pre-equilibrated to 40°C. The temperature of the oven was controlled by a loop control program written in Basic computer language. The program was designed to begin from the initial temperature of 40°C and stop at the final temperature of 88°C. The increment was set at 1°C, for every 4 hours, and the total reaction time was 8 days. Thus, an average rate of temperature increase of 0.25°C/hr was reached. Although the temperature increment was stepwise, it can be approximated as a linear increment because of the small temperature increment at every step. Samples were removed from the oven every 12 hours until all the data points were collected.

Data Analysis

Isothermal Kinetic Studies

Traditional first order kinetics and the Arrhenius temperature dependency was used to interpret degradation kinetics of thymopentin and its analogs.

Non-Isothermal Kinetic Studies

Both integral and derivative methods were utilized to analyze the non-isothermal stability data.

A. Integral Method. The nonisothermal computer program, NISO7 (Baxter Laboratories, McGraw Park, IL), was used to analyze the non-isothermal decomposition kinetic data. Theoretically, NISO7 uses the iterative simplex algorithm of Nelder and Mead to perform a least square to fit time, temperature and concentration data into an appropriate kinetic model. The following integral form of the first-order rate equation, equation 1, was used to fit the time, temperature and percentage of drug remaining.

$$C = Co \exp \int \exp\left(-\frac{Ea(t)}{RT}\right) \cdot dt \qquad (1)$$

The optimal values for the activation energy and the pre-exponential factor were obtained from the computer program. Before running the program, the first-order rate model was specified and the number of polynomial terms was determined within 3 to 20 terms so as to produce the best fit for the data. The initial estimated values for Ea and A were obtained from the isothermal preliminary experiments.

B. Derivative Method. A polynomial curve fit was involved in the derivative method. This curve fit was used to fit the plot of ln (% remaining) versus time, for non-isothermal data. The same polynomial term that was used in the integral method was chosen for the derivative method. The EZFIT® curve fit program (Noggle, J.H., U. of Delaware) was used to obtain the first derivative (dln(C)/dt) from the plot at different time points and, hence, corresponding to temperatures as well. The negative value for the first derivatives at each time points represents the rate constant (k) at each temperature. Therefore, the Arrhenius plot of ln (k) versus the reciprocal temperature could be performed, and the Ea and A values at various temperatures could be estimated from the slopes and intercepts.

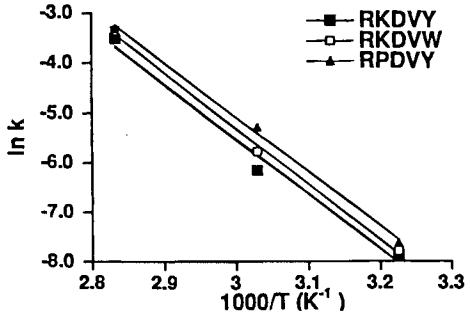

Fig. 1. The Arrhenius plot for the degradation of thymopentin (■ RKDVY) and its analogs (□ RKDVW and ▲ RPDVY) at pH 2 aqueous buffered solution.

RESULTS AND DISCUSSION

Reaction Order

The decomposition of thymopentin and its analogs (RKDVW and RPDVY) in aqueous solutions were plotted in a typical logarithm of percent remaining versus time. Linear relationships were observed for all studied peptides with correlation coefficients that were greater than 0.95. This indicated that the degradation of thymopentin and its analogs in aqueous buffered solutions followed apparent first-order degradation kinetics under the conditions studied. The slope of the plot represent the apparent first-order degradation rate constant (k_{obs}) at studied condition.

Effect of Temperature on Degradation

The effect of temperature on the degradation rate of thymopentin (RKDVY) and its analogs (RKDVW and RPDVY) was investigated at 37, 57 and 80°C in 0.05 M citrate buffer at pH 2 and $\mu = 0.5$. The degradation of thymopentin and its analogs followed a first-order degradation process at all temperatures and pH values studied. The degradation rate constants were summarized in Table 1. The degradation process was highly accelerated as temperatures increased. The rate constant for many chemical reactions can be empirically related to the absolute temperature by the Arrhenius equation. In this study, the dependence of the rate constant on temperature followed a linear Arrhenius plot, as can be seen in Fig. 1. The linear Arrhenius plot obtained here indicated that the degradation mechanism of thymopentin and its analogs were the same within the temperature range studied. The energy of activation (Ea value) at each pH was calculated from the slope of the Arrhenius plot, while the pre-exponential factor (A value) was calculated from the intercept of the plot. The shelf life ($t_{90\%}$) of thymopentin and its analogs at 25°C was determined by the equation:

$$T_{90} = 0.105/K_{obs\ 25°c} \quad (2)$$

where, k_{obs}(25°C) can be calculated by extrapolating the Arrhenius plot to 25°C or by using one of the rate constants from experimental temperatures. The above $t_{90\%}$ calculation was made on the assumption that the energy of activation remains constant over the temperature range of 25°C to 80°C. The calculated Ea, k_{obs} and $t_{90\%}$ at 25°C are given in Table 1.

Effect of Ionic Strength on Solution Stability

The influence of ionic strength on thymopentin stability was investigated at acidic (pH 2), neutral (pH 7.4) and basic pH values (pH 10). Table 2 lists the observed degradation rate constant (k_{obs}) of thymopentin in aqueous buffered solutions with various ionic strengths ($\mu = 0.02$, 0.1, 0.25, 0.5 and 1 in a pH 2 citrate buffer; $\mu = 0.04$, 0.1, and 0.5 in a pH 10 borate buffer; $\mu = 0.07$ and 0.28 in a pH 7.4 phosphate buffer) at 80°C. A modified Debye-Huckel equation (Equation 3) has been used to interpreted the influence of the buffer ionic strength on the reaction rate constant.

Table 1. First Order Rate Constants for Degradation of Thymopentin (RKDVY) and Its Analogs (RKDVW and RPDVY) at 37, 57 and 80°C

Peptide	pH	Kobs (hrs^{-1})				Ea Kcal/mole	T_{90}[b] (days)
		25°C × 10^{-5}[a]	37°C × 10^{-4}	57°C × 10^{-3}	80°C × 10^{-2}		
RKDVY	2	8.95	3.79	2.09	2.98	22.08	48.9
	4	9.78	3.47	1.66	1.60	19.38	44.8
	10	7.06	1.59	3.22	1.60	23.33	62.0
RKDVW	2	9.57	4.15	3.10	3.51	22.44	1.17
	10	8.79	3.32	1.18	1.85	20.34	2.06
RPDVY	2	11.7	4.86	5.07	3.64	21.82	37.5
	10	20.6	5.97	3.58	1.49	16.25	21.2

[a] First order rate constant (k25°C) predicted from rate constant at 37°C.
[b] Estimated from k 25°C, Ea: Energy of Activation.

Table 2. First Order Rate Constants for Degradation of Thymopentin in Various Ionic Strength (μ) Buffers, pH 2, 7.4, and 10 at 80°C

μ[a]	pH = 2 Kobs (hrs-1) 10^{-2}	μ	pH = 10 Kobs (hrs-1) × 10^{-3}	μ	pH = 7.4 Kobs (hrs-1) × 10^{-3}
0.02	2.46	0.04	5.11	0.07	8.21
0.1	2.39	0.1	4.98	0.28	7.67
0.25	2.48	0.5	5.96		
0.5	2.48				
1.0	2.45				

[a] μ = Ionic strength.

$$\ln Kobs = \ln Ko + 2QZ_AZ_B[\sqrt{\mu}/(1 + \sqrt{\mu})] \quad (3)$$

where k_o is the rate constant at $\mu = 0$, Q is a constant for a given solvent and temperature, and Z_A & Z_B are the charges on species A and B. According to the above equation, if the plot of ln (k_{obs}) versus $[\sqrt{\mu}/(1 + \sqrt{\mu})]$ yields a zero slope, then the effect of ionic strength on the degradation kinetics is considered to be negligible. A plot of ln (k_{obs}) vs. $[\sqrt{\mu}/(1 + \sqrt{\mu})]$ at pH 2, 7.4 yielded a slope approximately equal to zero. This result suggests that the degradation of thymopentin was independent of the buffer ionic strength in the pH and ionic strength ranges studied.

Effect of pH on Degradation

Over the time course of the studies, the degradation of thymopentin at various pH values followed apparent first-order kinetics. The pH-rate profile for thymopentin at a constant ionic strength (μ − 0.5) was generated to investigate the stability of thymopentin in the pH range of 2 to 10 at 80°C (Fig. 2). Thymopentin was found to be most stable in the pH range of 6 to 8. The data obtained from the pH-rate profile indicated that the orders, with respect to hydronium and hydroxide ions, were 0.144 and 0.169, respectively. Since the slope of the acidic or basic region of the pH-rate profile was neither +1 nor −1, the degradation of thymopentin did not undergo a specific acid or base catalysis reaction.

Several kinetic models can be proposed to describe the pH-rate profile for thymopentin. One particular kinetic model, which adequately describes the rate profile and is kinetically feasible, is presented here. The degradation kinetic model for the thymopentin pH rate profile is shown as follows:

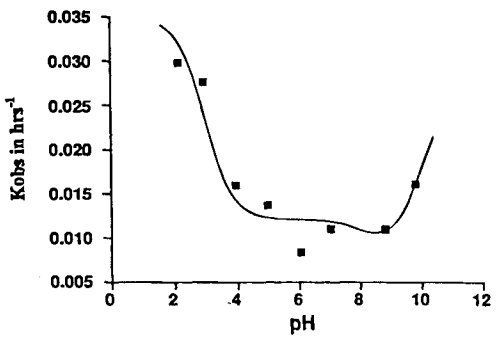

Fig. 2. pH rate profile for the degradation of thymopentin at 80°C. The solid line is the fitted line (Equation 5) and ■ are the experimental points.

$$\begin{array}{ccccccc} TH_4^{+3} & \xleftrightarrow{pKa_1} & TH_3^{+2} & \xleftrightarrow{pKa_2} & TH_2^{+1} & \xleftrightarrow{pKa_3} & TH_O \\ \downarrow k_1 & & \downarrow k_2 & & \downarrow k_3 & & \downarrow k_4 \end{array}$$

where TH_4, TH_3, TH_2, and TH represent four ionization species of thymopentin presented in a different pH range, k_{a1}, k_{a2} and k_{a3} are the ionization constants for thymopentin, and k_1, k_2, k_3 and k_4 are first-order rate constants for the reaction of TH_4, TH_3, TH_2 and TH species, respectively.

The shape of the pH-rate profile suggested that the overall rate of thymopentin degradation at a given pH can be expressed by the following equation:

$$k_{obs} = k_1[TH_4/T] + k_2[TH_3/T] + k_3[TH_2/T] + k_4[TH/T] \quad (4)$$

where $T = [TH_4] + [TH_3] = [TH_2] + [TH]$ and k_{obs} is the overall observed rate constant.

Equation [4] can then be rewritten as follows:

$$k_{obs} = \frac{k_1[H^+]^3 + k_2k_{a1}[H^+]^2 + k_3k_{a1}k_{a2}[H^+] + k_4k_{a1}k_{a2}k_{a3}}{[H^+]^3 + [H^+]^2k_{a1} + [H^+]k_{a2} + k_{a1}k_{a2}k_{a3}} \quad (5)$$

Using the PCNONLIN nonlinear curve fitting program, the experimental data were fitted into equation (5). It was found that the best nonlinear curve fitting could be obtained by using $k_{a1} = 1 \times 10^{-3}$, $k_{a2} = 1 \times 10^{-8}$ and $k_{a3} = 1 \times 10^{-10}$. These values were obtained theoretically and discussed in the next section. The calculated rate constants for thymopentin at 80°C k_1, k_2, k_3 and k_4 were found to be 34.9×10^{-3} hrs^{-1}, 12×10^{-3} hrs^{-1}, 9.5×10^{-3} hrs^{-1} and 26.0×10^{-3} hrs^{-1}, respectively. By substituting the rate constants into equation 5, the predicted pH-rate profile can be obtained. Figure 2 shows the predicted pH rate-profile for thymopentin. The line displayed in this figure represents the predicted theoretical curve and the data points represent the experimental results. The good agreement between the calculated values and the experimental data indicates that equation 5 is adequately used to describe the thymopentin degradation kinetics.

Non-Isothermal Kinetic Studies

The non-isothermal data for thymopentin and its analogs were used to calculate the Arrhenius parameters by using both integral and derivative methods. The following two examples explain how the integral and the derivative method was used to calculate the Arrhenius parameters for thymopendin at pH 2.

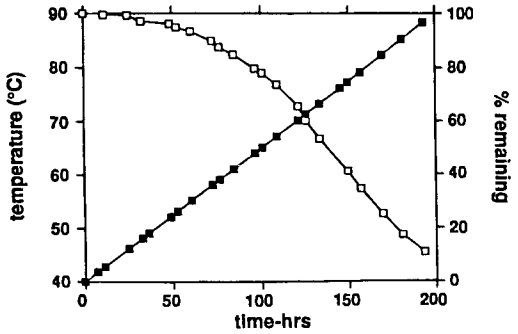

Fig. 3. Relationship between the temperature (■) and thymopentin percentage remaining (□) versus time, at a temperature increment of 0.25°C per hour (pH 2).

Integral Method

The NISO7 computer program was used to fit the degradation profile of thymopentin at pH 2. Figure 3 shows the double Y plot of the relationship between the temperature and peptide percentage remaining versus time. Polynomial terms ranging from 2 to 13 were selected for the curve fitting. It was found that five polynomial terms yielded the smallest standard deviation of residuals among any other number of polynomial terms and gave the best fit to the data. The output from the NISO7 program generated an optimal value both for the activation energy of 21.11 (Kcal/mole), and of 3.02×10^{11} (1/hrs) for the pre-exponential factor.

Derivative Method

The use of the derivative method to calculate the Arrhenius parameters was performed by plotting ln (% remaining) versus time. As in the integral method, five polynomial terms was used to fit the thymopentin degradation data. The Arrhenius plot was generated from calculated rate constants at corresponding temperatures (Fig. 4). Both the activation energy and the pre-exponential factor were calculated from the slope and intercept of the plot. The obtained Ea and A were equal to 20.80 (Kcal/mole) and 1.93×10^{11} (1/khrs), respectively.

Comparison of Isothermal and Non-Isothermal Methods

Table 3 lists a comparison of the Arrhenius parameters for thymopentin obtained from the isothermal and nonisothermal

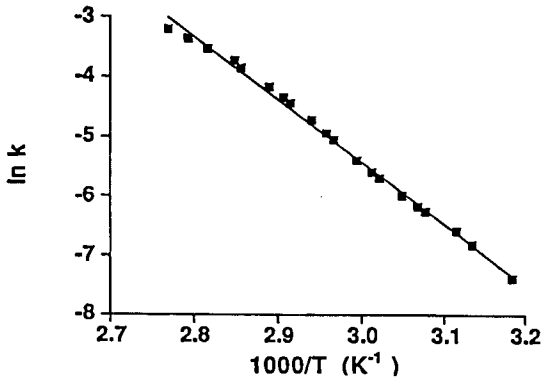

Fig. 4. Arrhenius plot for the degradation of thymopentin in pH 2 buffered aqueous solution at the corrrresponding temperature generated from the nonisothermal method.

Table 3. Comparison of the Activation Energy Obtained by the Isothermal and Nonisothermal Method of Thymopentin (RKDVY) and Its Analogs (RKDVW and RPDVY)

Peptide	pH	Activation energy, Ea, Kcal/mole		Percent difference[a]
		Isothermal	Nonisothermal	
RKDVY	2	22.08	21.11	+4.39
	4	19.38	19.99	−3.15
RKDVW	2	22.44	22.98	+6.51
RPDVY	2	21.82	22.99	−5.35
	10	16.25	15.46	+4.87

[a] Difference = (Isothermal − Nonisothermal) * 100 / Isothermal.

kinetic studies in pH 2 buffered solution. A good agreement in the values for the energy of activation was found from both isothermal and nonisothermal studies. The values of percentage difference for the activation energies were found to be less than 7%. The Arrhenius factor values from the nonisothermal studies were somewhat underestimated. For example, the Arrhenius factor values at pH 2 and a $\mu = 0.5$ were found to be 3.02×10^{11} by the integral method, 1.93×10^{11} by the derivative method and 1.19×10^{12} by the isothermal method. The values of the percentage difference for the Arrhenius factor is high because this is estimated from the intercept of the Arrhenius plot in the isothermal studies. Thus, a slight variation in slope of the line will affect the intercept considerably. The shelf life estimated from the isothermal study was 48.9 days. According to the nonisothermal study, the estimated shelf life, based on the integral method and the derivative method, was 45.9 days and 45.4 days, respectively.

These results indicate that the non-isothermal methodology employed in this investigation may be used to extrapolate the peptide stability and, therefore, to estimate the shelf-life for pharmaceutical preparation of other peptides. Thus, the non-isothermal procedure provides an attractive alternative to the isothermal method, as it requires a much lower amount of both material and time, compared to the isothermal stability study.

Development of thymopentin analogs with higher stability and longer half-life is desired with the purpose of increasing its circulation time *in vivo* and maximizing its therapeutic effect. The analogs used in the present investigation, RKDVW and RPDVY are attempts to increase the stability of thymopentin by chemical modification. The aminoacid proline in thymopentin was changed for lysine yielding RPDVY. The degradation of RPDVY in plasma was found to be three times slower than thymopentin probably by changing enzyme affinity. Other analogs such as RKNVY which exploits the deamidation reaction of Asn yielding thymopentin have been studied using the methodology reported in this investigation. Conversion of RKNVY to RKDVY prolonged the circulation of thymopentin for a period of one hour.

In conclusion, these results indicate that the non-isothermal methodology provides an attractive alternative to the isothermal method, as it requires a much lower amount of both material and time, to determine the peptide stability and to estimate the shelf-life for peptide pharmaceutical preparation.

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