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ACCELERATED STABILITY TESTING OF PROTEINS AND PEPTIDES: pH-STABILITY PROFILE OF INSULINOTROPIN USING TRADITIONAL ARRHENIUS AND NON-LINEAR FITTING ANALYSIS

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ABSTRACT

The stability of insulinotropin was evaluated as a function of pH and temperature in the present study so as to predict stability at ambient and subambient temperatures. For traditional Arrhenius analysis, there is generally a loss of statistical information for the estimates of the energy of activation (E_a) and the frequency factor (A) due to the use of two separate regression steps. Therefore, a comparison was made between traditional Arrhenius analysis and a non-linear fitting approach utilizing

$$\log (C/C_0) = -0.434\ t\ A \exp(-E_a/RT)$$

where C is the concentration, 0 denotes the initial value, t is time, T is temperature, and R is the gas constant. A good agreement was found in the estimates by the two methods; however, the nonlinear method did provide an improvement in confidence limits under some conditions. Arrhenius plots for the degradation of the peptide appear to obey the Arrhenius equation from 5 to 50°C under alkaline and acidic conditions; however, some deviation was observed at neutral pH. The analysis predicts that at 25°C, maximal stability for the peptide is at approximately pH 8. Both methods predict 10% loss of potency in 4-5 months at pH 7 and 25°C, which appears to be reasonably consistent with ongoing real time studies.

INTRODUCTION

Accelerated stability testing at elevated temperatures is an accepted practice in the pharmaceutical industry due to development time tables which typically do not allow real-time testing for periods of two years or more. However, the advantage of speed is not without risk. Curved of Arrhenius plots are often observed due to changes in mechanism, rate determining step, and changes in heat capacity of the reactants or activated complex. For proteins and peptides, the situation is further complicated by temperature induced alterations in higher order structure. Generally an investigator must accept these risks early in the drug development process. A final negative aspect to standard Arrhenius analysis is the wide confidence intervals for estimated rate constants which result from the use of two linear regression steps in series; the first to obtain the rate constant, and the second being the Arrhenius plot itself. An approach which improves the confidence interval for the estimated parameters has been recommended by King et al. (1) and Ertel and Carstensen (2). This approach has been applied to a peptidic drug in the present study.

Insulinotropin is a 31 amino acid peptide being investigated for use in Type II diabetes (3). We have been studying the chemical and physical properties of insulinotropin so as to support formulation development. Since a wide variety of routes of administration and formulations will be investigated for this drug, it is imperative that a thorough understanding of the solution stability of insulinotropin be acquired. The main objectives of the present study were as follows: First, to investigate the stability as a function of pH and temperature. Second, to use Arrhenius analysis to describe the above relationships and help predict stability at ambient and subambient temperatures. Last, to determine the utility of nonlinear regression fitting of the Arrhenius model and compare it to the traditional Arrhenius approach.

EXPERIMENTAL

The insulinotropin utilized in the present study was produced by solid state synthesis by Bachem Feinchemikalien AG (Switzerland) and was greater than 99% pure excluding volatiles. The buffer solutions prepared for the study utilized 50 mM citrate (pH 3), phosphate (pH 7 and 8), or borate (pH 9 and 10), with the ionic strength kept constant at 0.15 M using sodium chloride (calculated by reference 4). The only exception was the pH 6 citrate buffer which utilized 30 mM citrate. Solutions at pH 4 and 5 were not included for solubility reasons. The above buffers with 0.100 mg/mL of drug were sterile filtered (0.2 μ Millex GV filter, Millipore) into autoclaved Eppendorf tubes (approximately 500 μl/tube). The tubes were then kept at the appropriate

temperature ranging from 5 to 70°C. Preliminary data at 40°C indicated that insulinotropin had poor stability in the extreme pHs at higher temperatures (degradation kinetics too fast to monitor effectively) and therefore, only the neutral pHs were studied at 70°C. The loss of insulinotropin was determined as a function of time using reverse-phase chromatography with external standards. The chromatography columns included a Vydac C4 guard column and a Vydac C4 analytical column (25 cm X 4.6 mm). Detection was at 214 nm. The gradient utilized a 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The gradient program held B at 25% for 5 minutes, and then linearly ramped to 50% B in 25 minutes. B was held at 50% for an additional 5 minutes prior to reequilibration at 25% B.

THEORETICAL

Traditional Arrhenius analysis involves plotting the logarithm of the rate constant versus reciprocal temperature using Equation 1

$$\ln k = \ln A - \frac{E_a}{RT} \qquad (1)$$

where R is the gas constant, E_a is the energy of activation, and A is the frequency factor. Typically, the rate constants are derived from separate plots of concentration as a function of time at several elevated temperatures. A major flaw of the traditional Arrhenius approach is that it utilizes two sequential linear regression steps. In particular, the errors associated with the determination of the rate constant are not taken into account. This results in a loss of statistical power for the estimates of the E_a and log A parameters. King et al. (1) have developed equations which allow for the direct prediction of shelf-life based on concentration, time, and temperature data. They suggest this leads to an improved shelf-life estimate, and smaller and more symmetrical 95% confidence intervals.

The nonlinear model described by King et al. (1) was developed by substituting the appropriate rate equation (in this case a first-order reaction equation)

$$\log \frac{C}{C_o} = - \frac{kt}{2.303} \qquad (2)$$

directly into the Arrhenius equation (Eq. 1), yielding

$$\log \frac{C}{C_o} = -\frac{tAe^{-\frac{E_a}{RT}}}{2.303} \quad (3)$$

A nonlinear regression fitting program, PCNONLIN (SCI Software, Lexington, KY), was used to fit log C/C_0, time, and temperature data. Theoretically, this method will give better statistical value to E_a and log A estimates because it uses the raw data directly, without going through two linear regression steps as in the case with the traditional Arrhenius approach.

RESULTS AND DISCUSSION

Log concentration versus time plots were generated for every pH and temperature studied. Linear regression analysis was utilized to determine the first-order rate constants, which were then plotted by the traditional Arrhenius approach (see Figure 1 for a typical example). The Arrhenius plots appeared to be reasonably linear for pHs 3 and 9. At pH 10, only two temperatures were studied and, thus, no information was gained on the linearity of the plot. Some upward curvature was suggested in the neutral pH (6-8) plots (see Figure 2). The curvature may be due to a change in E_a as a function of temperature (5), or due to altered higher order structure in the peptide as a function of temperature (6,7). Hydrolysis, one of the major degradation pathways for proteins, typically yields a V- or U-shaped curve due to the combination of specific acid catalysis, specific base catalysis, and direct attack by water (8). It is likely that the minimum in the pH-stability curve will involve all 3 mechanisms to some extent. Thus, the possibility of curvature is increased in the neutral pH region since three separate activation energies may be involved.

The fit of the raw data using Equation 3 by the nonlinear model is shown in Figures 3 and 4 for the pH 3 and 8 data, respectively. The fit was very good for the pH 3 data, but did not describe the pH 8 data well. The fit of the pH 6 and 7 data also appeared to be poor. This result is consistent with the nonlinearity observed in the traditional Arrhenius plots of the same data in the neutral pH region (e.g., Fig. 2). A good fit can not be expected if the model used is incorrect. The frequency factor and energy of activation as determined by the traditional and nonlinear Arrhenius approaches are compared in Table 1. The values obtained by the two methods are all within 5% of each other for both parameters.

Some interesting differences were observed between the two analyses in terms of the confidence intervals of the estimated log A and E_a values (Table 2). The confidence intervals were very similar for the two methods at pH 3 and pH 9. This would seem to suggest that the nonlinear method offers little advantage in cases where a reasonable number of time and temperature points

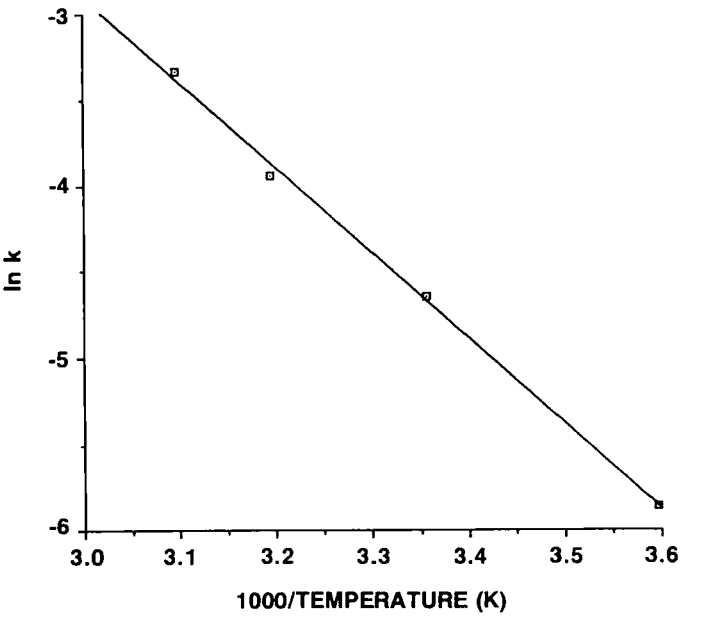

FIGURE 1. Traditional Arrhenius plot for degradation of insulinotropin in a pH 3 citrate buffer (5-50°C).

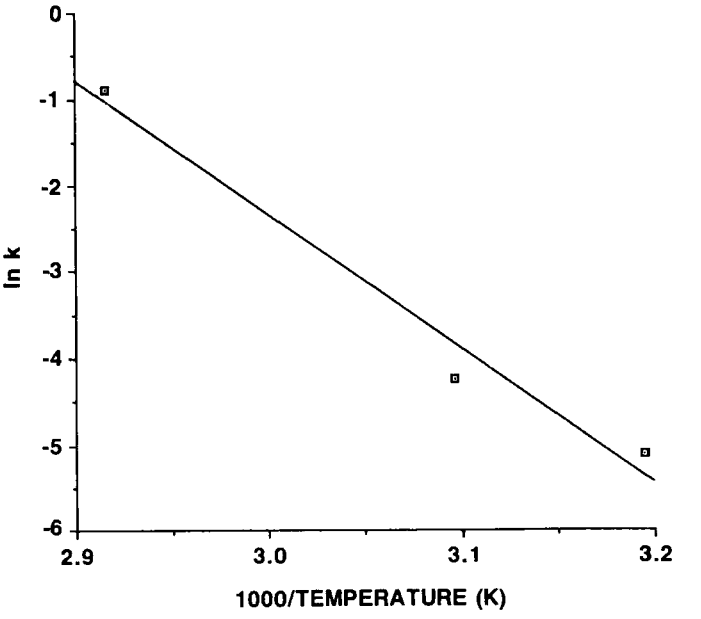

FIGURE 2. Traditional Arrhenius plot for degradation of insulinotropin in a pH 8 phosphate buffer (40-70°C), showing slight upward curvature.

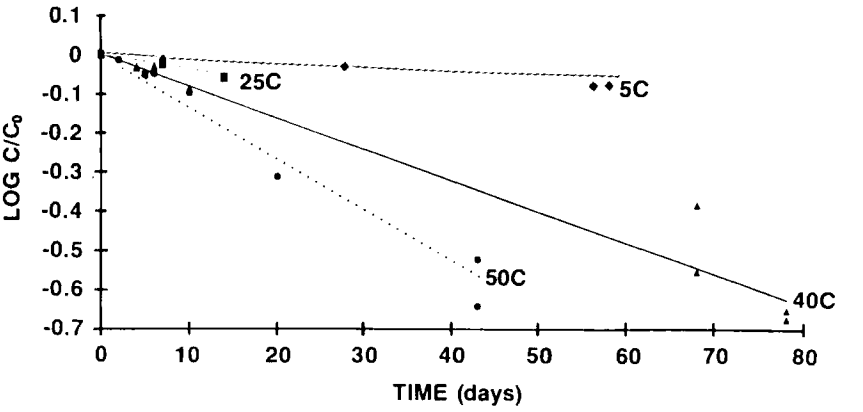

FIGURE 3. First order degradation plot for loss of insulinotropin at pH 3. The lines represent the calculated values using the nonlinear fit method (Equation 3).

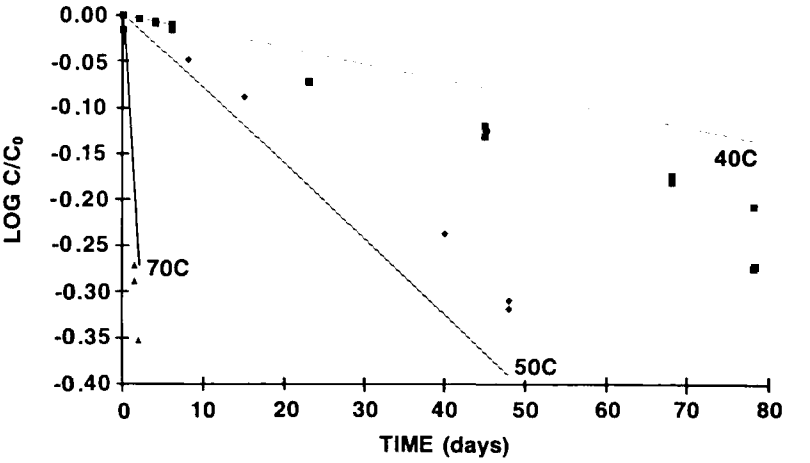

FIGURE 4. First order degradation plot for loss of insulinotropin at pH 8. The lines represent the calculated values using the nonlinear fit method (Equation 3).

TABLE 1

Arrhenius and Nonlinear Calculated Log A and E_a Values

	log A ($days^{-1}$)		E_a (kcal/mol)	
pH	Nonlinear	Arrhenius	Nonlinear	Arrhenius
3	5.24	5.17	9.99	9.84
6	10.32	10.25	17.99	18.04
7	13.17	13.03	22.16	22.06
8	19.31	19.11	31.11	30.79
9	8.81	8.73	15.00	14.92
10	16.58	15.89	25.00	24.04

TABLE 2

95% Univariate Confidence Intervals

	Log A ($days^{-1}$)		E_a (kcal/mol)	
pH	Nonlinear	Arrhenius	Nonlinear	Arrhenius
3	4.1 to 6.4	4.1 to 6.2	8.3 to 11.7	8.5 to 11.2
6	9.2 to 11.5	-16.5 to 36.9	16.2 to 19.8	-57.8 to 21.7
7	11.6 to 14.7	-24.9 to 50.8	19.7 to 24.6	-78.5 to 34.6
8	17.5 to 21.1	-22.3 to 60.4	28.3 to 34.0	-92.4 to 31.0
9	7.9 to 9.7	7.8 to 9.7	13.7 to 16.3	13.6 to 16.3
10	15.8 to 17.4	---	23.8 to 26.2	---

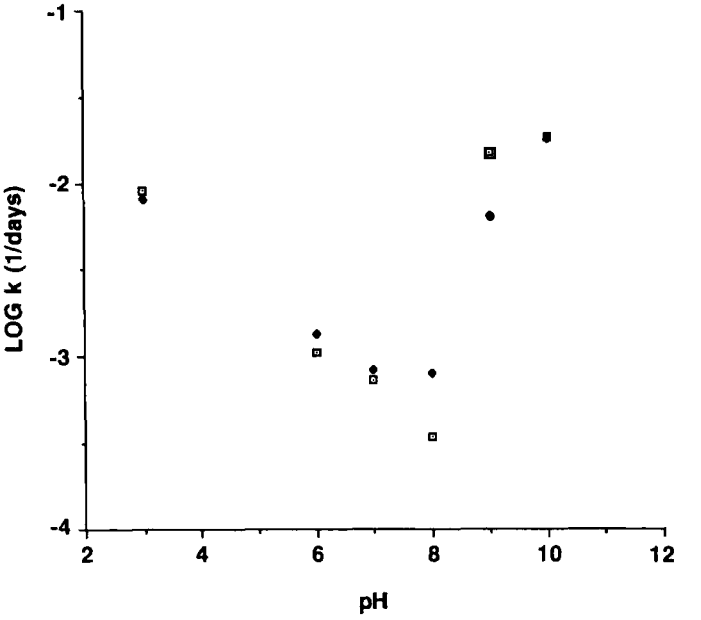

FIGURE 5. Estimated pH-stability plot at 25°C using the parameters obtained for Equations 1 (□) and 3 (•).

TABLE 3

Comparison of Predicted and Observed Potency

Potency at pH 7 after 12 weeks	5°C	25°C
Observed	95.5%	90.3%
Traditional Estimate	99.6%	94.2%
Non-linear Estimate	99.5%	93.3%

are evaluated. However, at the neutral pHs, a dramatic difference is seen. The traditional method has extremely broad confidence intervals for the log A and E_a estimates, whereas, the confidence interval for the nonlinear method was much narrower. Thus, it appears advantageous to utilize the nonlinear method when a limited number of temperature points are utilized in an accelerated study.

At 5 and 25°C, insulinotropin is fairly stable in neutral pH. Since real time stability studies under these conditions would take years to complete, it was necessary to utilize accelerated stability studies. An estimated pH-stability profile at 25°C was generated using both the traditional and nonlinear Arrhenius estimates (Figure 5). Both curves show reasonable agreement, and suggest maximal stability at approximately pH 8. A comparison of predicted and the observed potency for a real time sample at pH 7 for 12 weeks is shown in Table 3. The predicted values appear to slightly underestimate the degradation rate, possibly due to the observed upward curvature in the Arrhenius plots in the neutral pH region. Nonetheless, both methods provide reasonable estimates for formulation development.

ACKNOWLEDGEMENTS

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