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Evaluation of Cholecystokinin (CCK-8) Peptide Thermal Stability for Use as Radiopharmaceutical by Means Isothermal and Nonisothermal Approaches

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ABSTRACT The purpose of this research was to study thethermal stability of cholecystokinin octapeptide (CCK-8) in aqueous solution at pH 12 and ionic strength 0.01M, which were kept as constants, by using isothermal and nonisothermal methods.

The isothermal decomposition of CCK-8 was investigated as a function of temperature (40°C to 70°C). Nonisothermal stability studies were performed using a linear increasing temperature program. Two different nonisothermal studies were carried out at 0.25°K and 0.5°K per hour, and the temperature interval varied from 40°C to 82°C.

The degradation of CCK-8 followed first-order kinetics, obeying the Arrhenius equation in the experimental temperature range. This indicated that the degradation mechanism of CCK-8 could be the equal within the temperature range studied. The nonisothermal approach resulted in activation energy (Ea) and shelf-life ($t_{90\%}$) values that agree well with those obtained by the isothermal method. The level of uncertainty in the estimates of $t_{90\%}$ and Ea values is determined mainly by the extent of drug degradation and temperature change during the experiment. Therefore, nonisothermal experiments save time, labor and materials (i.e. the amount of drugs necessary to conduct the experiment) compared to the classic isothermal experiments, if they are performed using a suitable experimental design and a precise analytical method.

KEYWORDS Cholecystokinin, Stability, Nonisothermal approach, HPLC, Shelf life

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INTRODUCTION

The proliferation of new peptides and proteins requiring characterization is a direct result of recent advances in genomics and proteomics. However, stability is a particular problem in the biotechnology industry because peptides and

proteins efficacy as therapeutic or diagnostic agents can be affected during preparation, shipping, and storage.

Determination of the drug degradation kinetics and drug stability at ambient temperature can be time-consuming because chemical reactions proceed relatively slowly at low temperatures. Undoubtedly, accelerated testing at higher temperatures permits a significant reduction in testing time; however, preparation and assay of the large number of samples necessary for the multiple-temperature accelerated test may offset this benefit.

The method of nonisothermal prediction of degradation rate was therefore developed to reduce the experimental effort. This is achieved by allowing kinetic parameters to be estimated from a single set of drug concentration vs. time data which is obtained while temperature changes as a function of time according to some algorithm (Waltersson & Lundgren, 1983; Kipp et al., 1986; Zhan et al., 1997a,b).

Peptide-based radiopharmaceuticals were introduced into clinical work in the early 1990s. For in vivo use as a radio pharmaceutical, the natural peptide is modified in order to enhance its metabolic stability and allow stable labelling, but preserving at the same time its binding affinity for the target receptor (McAfee & Neumann, 1996; Liu et al., 1999; Gotthard et al., 2004; Laverman et al., 2004). A broad spectrum of radiolabel peptides with high affinity for receptors expressed in tumor cells is currently under preclinical and clinical investigation for scintigraphic imaging and radionuclide therapy (Laverman et al., 2004). Examples include cholecystokinins (CCK), a family of neuropeptides whose members contain a number of different amino-acid residues that differ in their specificity for the CCK receptors (Lydiard, 1994). For example, the smaller forms, CCK-4, CCK-5 (pentagastrin) or CCK-8 have specificity for the CCK-B receptor (Laverman et al., 2004). High-affinity receptors for these peptides are overexpressed in several kinds of human tumors, and may therefore represent new molecular targets for cancer diagnosis and therapy (Matsumori et al., 1995; Okarvi, 1999; Behr et al., 1999; Goetze & Rehfeld, 2003; Reubi et al., 2004).

The octapeptide CCK-8, which constitutes the 26–33 segment of the hormone cholecystokinin, is used for the diagnosis of CCK-B receptor-expressing tumors after labelling preferably with technetium (99m) radionuclide (Laverman et al., 2004). The rapid pharmacokinetics of these peptides is well matched to the physical half-life of technetium (Tc) (99m), and efforts have been focused on developing suitable radiolabelling procedures. However, Tc (99m)-labelling yields depend on reaction time, pH and temperature, which may all affect the biological properties of the peptide, impeding receptor binding potential (Rossin et al., 2001; Arano, 2002).

Despite the interest in cholecystokinin fragments and their applications in nuclear medicine, little has been published so far on the stability of cholecystokinin fragments, especially before the labelling process. The aim of this work is to evaluate the chemical stability of CCK-8 in aqueous solution as a function of temperature, with pH level and ionic strength kept as constants before labelling, in order to reduce untoward effects that could modify their efficacy as therapeutic or diagnostic agents. Isothermal and nonisothermal methods were used to achieve this purpose. In addition, the effects of the conditions of nonisothermal experiments on the accuracy of kinetic parameter estimates were studied, particularly with regard to experiment duration and temperature range.

MATERIALS AND METHODS

Materials

Nonsulphated CCK-8 (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2) was purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and reagents were analytical or HPLC grade. Deionised water was purified in a MilliQ plus system® (Millipore, Molsheim, France) prior to use.

HPLC Method Validation and Development

The chromatographic system used was a Waters apparatus (Milford, MA, USA) consisting of a pump, 600E multisolvent delivery system, 700 wisp sample processor, a Nova Pack C-18 column (150x3.9 mm, 60 Å, 4 μm particle size, Waters), and a 2487 programmable multiwavelength detector. The data collection and analysis were performed using the Millenium32® chromatography program (Waters).

The mobile phase was an acetonitrile-water (30:70, v/v) mixture with 0.05% trifluoroacetic acid added to improve peak separation, at a flow rate of 1.0 mL/min. for 9 min., and the injection volume was 25 μL. The

detection wavelength was set at 280 nm. All solvents were filtered with 0.45 μm (pore size) filters (Millipore) and degassed.

The RP-HPLC method was validated according to the International Conference on Harmonization (ICH) Guidelines (ICH, 1996); the factors considered included selectivity (α=1.65) and resolution ($Rs > 1.4$) in the identification step (Fig. 1a). For assay precision and accuracy, three replicates each of low (6 μg/mL), middle (12 μg/ml) and high (18 μg/mL) standard samples were analyzed on three different days over a period of one week. The interday precision, expressed as residual standard deviation (RSD), varied from 1.62 to 2.11%, while the intraday precision fluctuated between 1.28 and 2.17%. The accuracy of the system, determined using the standard addition method, ranged from 95.9 to 103.6 with a mean of 98.6% and an RSD of 2.35%. In this study, the linearity of peak area versus prepared concentration of peptide (μg/mL) was examined. For this study, five standard curves with six standard samples each in the range of 6 to18 μg/mL were prepared on three separate days. The regression line calculated using the least square method was: Peak Area (μV·s) = (5649 ± 1256) + (8268 ± 105) concentration with the confidence intervals calculated at $P = 0.05$. The determination coefficient was $r^2 = 0.9955$ and the coefficient of variation was 2.45%. ANOVA analysis was used to confirm the linearity of the method through the rejection of the null hypothesis of deviation of the linearity for a significance level of 0.05 ($F_{(4, 24)} = 2.38$, $P > 0.05$).

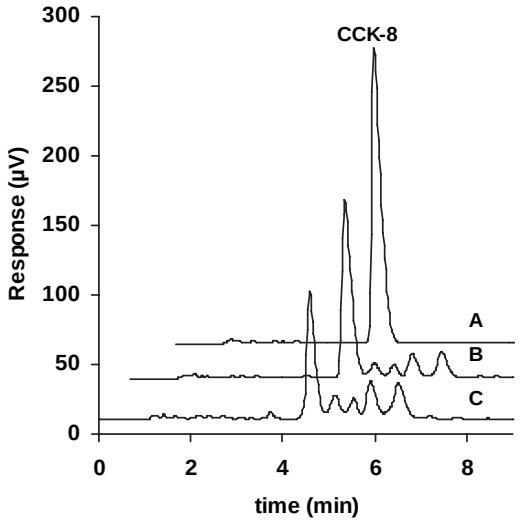

FIGURE 1 **Chromatograms of CCK-8: (A) Standard Sample; (B) Sample Stored Under Isothermal Conditions (60°C, 72 h); (C) Sample Stored Under Nonisothermal Conditions (76°C, 144 h).**

The detection and quantitation limits were calculated by the method based on the standard deviation (S.D.) of the response and the slope of the calibration curve (S), according to the following formulas: LOD = 3.3(S.D./S) and LOQ = 10(S.D./S), where the S.D. of the response is determined from the residual sum of squares of the regression line. The limits were 0.96 and 2.92 μg/mL respectively, lower than the smallest concentration in the linearity range. In addition, acceptable robustness was observed, indicating that the method remains unaffected by small but deliberate variations as described in the ICH guidelines.

Stability Studies

Isothermal Study

The oven (Model BR-UT 6000; Heroes Instruments, Hanau, Germany) temperature was preset and maintained at the desired temperature for isothermal studies. A 1-mg/mL peptide solution in dimethyl sulphoxide (DMSO) was mixed with NaOH solution to yield a final bulk concentration of 100 μg/mL, at pH 12 and ionic strength 0.01M. Aliquots of this bulk solution were stored in the oven at fixed temperatures (40, 50, 60, and 70°C) with variations of less than ± 0.1°C. Aliquots were removed from the oven at various time intervals, diluted with the mobile phase in order to obtain concentration values within the calibration range, and analyzed the same day in triplicate.

Nonisothermal Study

The temperature of the oven was controlled by a loop-control program written in Test Point® (version 4.0) computer language. With this program, it is possible to use different heating control systems (linear, reciprocal, logarithmic or exponential).

As a preliminary step, the oven temperature was preequilibrated at 40°C. Thus, the program was set to begin from the initial temperature of 40°C and stop at the final temperature of 82°C. The increment was set at 0.25°K per hour, and the total reaction time was 7 days. Additionally, two experiments were conducted in the same temperature range as under isothermal conditions, but with a reduced experimental period. For these experiments, the heating rate was increased from 0.25 °K to 0.5°K per hour.

Samples were prepared as in isothermal studies, and they were removed from the oven every 12 hours

until all the data points were collected. Unless otherwise indicated, all the samples were analyzed in triplicate.

Data Analysis

Isothermal Studies

Traditional first-order kinetics and the Arrhenius temperature dependency (Eq. 1) were used to interpret the degradation kinetics of the CCK-8 fragment.

$$k = A \cdot \exp^{(-Ea/RT)} \tag{1}$$

where k is the rate constant, A is the frequency factor, Ea is the activation energy, R is the gas constant and T is the absolute temperature.

Nonisothermal Studies

The complexity of a nonisothermal study is primarily related to the temperature-rise program and the associated data-treatment method (Kipp, 1985; Zhan et al., 1997a,b).

Recent developments in the field have lead to advances in both temperature-control systems and computation methods for nonisothermal experiments. As a result of these improvements, a linear heating model has become feasible for use in this study. Therefore, a linear heating model was chosen for its simplicity and its convenience.

A linear temperature program can be described by Eq. 2:

$$T_i = T_0 + vt \tag{2}$$

where v is the heating-rate constant, and T_0 and T_i are the temperatures (°C) at initial time and at time t, respectively.

Degradation of a drug can be described by the following equation:

$$-(dC/dt)_i = k \cdot C_i^n \tag{3}$$

where C_i is the drug concentration at time t_i, k is the rate constant and n is the order of the reaction. For a first-order reaction (n=1), a combination of the integrated rate expression and the Arrhenius equation yields:

$$C = C_0 \cdot \exp^{-A \cdot \int_0^t \exp^{[-Ea/RT(t)]}dt} \tag{4}$$

The integral method involves direct evaluation of the integrated expression on the right side of the equation in Eq. 4. The Nonlinear Fit function from the Mathematica® program (Wolfram, 1988) was used for this evaluation. The Nonlinear Fit function allows a direct nonlinear estimation of the activation energy (Ea), the frequency factor (A) and the initial drug concentration (Co) to be expressed as a percentage of the drug remaining. The maximum number of iterations in the search was 30 and the Levenberg-Marquardt method was used for the minimization of the χ^2 merit function. The estimates of the model parameters were chosen to minimize the χ^2 function given by the sum of the residuals' squares. Because the optimization methods used by Nonlinear Fit function are iterative, starting values are required for the parameter estimate search. Careful choice of starting values may be necessary, as the parameter estimates found by Nonlinear Fit may represent a local minimum in the χ^2 function. Therefore, the initial estimated values for Ea and A which had been obtained from the preliminary isothermal experiments were used as starting values in order to converge much faster to a solution.

RESULTS AND DISCUSSION

Under isothermal conditions, temperatures higher than 70°C were ruled out because it was not possible to follow the degradation process at those temperatures. A first-order degradation process was observed at all temperatures that were studied. The rate constants were obtained from the slopes of the semi-log plots of concentration versus time by linear regression analysis. The correlation coefficient was greater than 0.96. Thus, the rate constants were evaluated at several temperatures, T_i, and the Arrhenius parameters were determined in the usual manner using Eq. 1 in its logarithmic form. The dependence of the rate constant on temperature followed a linear Arrhenius plot (r = 0.9996), indicating that the CCK-8 degradation mechanism remained constant within the studied

temperature range. This observation confirmed the HPLC results because similar chromatographic behavior was observed at all temperatures (Figs. 1b, 1c). The Ea and A were 24.5 Kcal/mol and 1.07×10^{14} h^{-1}, respectively. The estimated shelf-life ($t_{90\%}$), i.e. the time period necessary for a drug to degrade to 90% remaining at 25°C, was 39 days. This calculation was made on the assumption that the activation energy remains constant throughout the temperature range from 25°C to 70°C.

Like the traditional isothermal stability study, the nonisothermal experiment is also based on the Arrhenius equation, but its accuracy and precision of the estimates depend largely on the experimental conditions, such as the experimental period and temperature change and the data-analysis method (Kipp, 1985; Yoshioka & Uchiyama, 1987; Zhan et al., 1997a, b). Thus, to obtain accurate results, the drug degradation must be as uniform and complete as possible within the experimental temperature range, and the mean temperature must be kept as close as possible to room temperature. Initially, the experimental period was fixed at 7 days, as is done in slower experiments under isothermal conditions, whereas the temperature range was set up to be from 40°C to 82°C, in order to obtain a linear heating rate of 0.25°K per hour and facilitate greater degradation.

When conducting the data analysis, the two most popular approaches, derivative and integral, can both be used to calculate the Arrhenius parameters. If the first method is employed, the concentration data are transformed by a function dependent on reaction order. The resulting function is fitted to a power series with time and its derivative is provided a rate constant at each temperature. Ea and A are calculated from the slope and intercept of the Arrhenius plot, respectively, but their values depend on the number of terms and the kinetic model used (data not shown). Several statistical criteria can be applied to the data in order to choose the best model. For example, Kipp (1985) proposed that the goodness of fit is customarily estimated by the coefficient of linear correlation. However, this parameter is subject to random fluctuations and its unreliability must be taken into account by setting confidence limits. Within these limits, all models are equally probable. The second method involves a direct nonlinear estimation of the Arrhenius parameters from Eq. 4 without preliminary mathematical treatment; thus providing reliable estimates with smaller deviations and biases (Yoshioka & Uchiyama, 1987). In our study, Mathematica ® was used to fit the degradation profile of CCK-8. Both Ea and A were determined by an iterative nonlinear least square method in accordance with the criteria put forth in the "Materials and Methods" section. This approach is known to be very sensitive to the reaction order. An analysis of the residuals of the nonlinear fitting results provided a clear indication of the best model; therefore, prior knowledge of the reaction order was not necessary.

Fig. 2 shows the relationship between the remaining peptide percentages vs. time as a function of the heating rate. The results were obtained by using Mathematica® to fit the degradation profile of CCK-8. Analysis of the nonlinear fitting results provides a clear indication that first-order kinetics is the best model (see Table 1) because it had the lowest estimated variance ($s^2 = 0.427$, $n = 45$), approximately 30 times lower than for zero-order kinetics ($s^2 = 11.62$). This value increased up to 300 times for second-order kinetics. The estimated parameter values for zero-order or second-order kinetics are not congruent with the isothermal values. It should be remembered that the prime objective of kinetic analysis is to obtain information with physical sense.

A good agreement in the activation energy values was found between the isothermal and nonisothermal

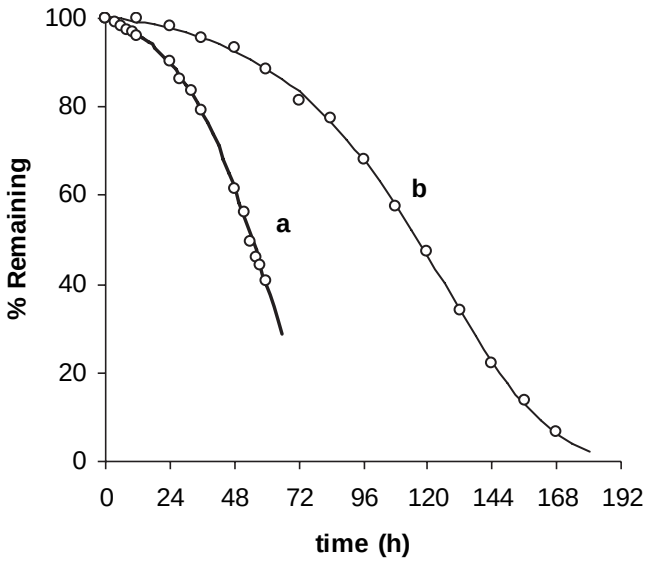

FIGURE 2 **Variation of CCK-8 Percentage Remaining Versus Time as a Function of the Nonisothermal Experimental Conditions: Temperature Change and Experimental Period: (a) 40°C –70°C, 60h; (b) 40°C –82°C, 168 h.**

TABLE 1 **Comparison of the Activation Energy (Ea) and Frequency Factor (A) with 95% Confidence Intervals (in brackets), Obtained by Nonisothermal Integral Approaches as a Function of the Reaction Order and the Isothermal Approach**

Nonisothermal	Ea (cal/mol)	Ln(A /h^{-1})
Zero-order	9269 [7890–10648]	13.38 [11.33–15.42]
First-order	24077 [23628–24526]	31.47 [30.81–32.15]
Second-order	63449 [50263–68634]	85.28 [77.84–92.72]
Isothermal	24518 [22792–26244]	32.30 [29.64–34.95]

methods, assuming first-order kinetics. The percentage difference in activation energies was observed to be less than 2%, whereas the difference was 56.4% for the frequency factor. This should be interpreted as a high–but nevertheless congruent–value because, in the isothermal approach, the frequency factor (A) was calculated from the intercept of the Arrhenius plot where a slight variation in slope would affect the intercept considerably. Similar results were obtained during the decomposition of thymopentin and its analogs in aqueous solution (Mu-Lan & Stavchansky, 1998). In that study, the estimated shelf life, assuming first-order kinetics, was 42.2 days, a value that was slightly higher than, but still comparable to, the value obtained from isothermal data.

However, the similarity of the Ea values that were obtained under isothermal and nonisothermal conditions suggests that a similar mechanism and similar kinetics were in effect, despite the fact that isothermal and nonisothermal experiments were conducted at different temperature ranges.

At ambient temperature, the data show that CCK-8 fragment is stable before the labelling process for at least 40 days, without compromising its capacity for subsequent receptor binding. Stability can also be prolonged for up to two years if the sample is stored at 5°C. To arrive at this result, constant activation energy over the temperature range of 5°C to 70°C was assumed. However, a real-time stability experiment should be performed in the future to confirm these data.

Two experiments were conducted in order to evaluate the effects of nonisothermal experimental conditions on the accuracy of kinetic parameter estimates. These experiments were conducted in the same temperature range as was used under isothermal conditions; however, the experimental period was reduced (see Table 2). The results indicate that the estimates of Ea were less accurate (i.e., far from the isothermal value) and less precise (i.e., small number of experimental points and increased error variance) when the experimental period was shortened. Furthermore, the extent of drug degradation was lower. Also, a marked difference in the $t_{90\%}$ estimates was observed, even when the final degradation ratios were approximately equivalent. t first, in the same temperature range

According to the Arrhenius equation, the kinetic parameters should have been equivalent while they were both in the same temperature range because both parameters were considered to be independent of temperature (see Table 2). Assuming that temperature was well-controlled and that it rose exactly as it had been programmed to (the difference between the theoretical and experimental heating-rate constant was less than 1.6%), the significant discrepancy between the estimates may be attributed to errors committed in measuring drug content, and also to the strong interrelationship between the kinetic parameters, as shown by the asymptotic correlation matrix. Thus, small variations in drug concentration could affect the kinetic parameters, and therefore influence the $t_{90\%}$ changes. In our study, the discrepancies were more likely to be caused by the strong interdependence of the kinetic parameters rather than the variations in the analytical method, considering that the coefficient of variation was lower than 2.5%. Moreover, it is possible in practice to envision a

TABLE 2 **Effect of Final Percentage Degraded, Temperature Range and Experimental Period on Precision of Estimates for Activation Energy (Ea) and Shelf Life ($t_{90\%}$), With 95% Confidence Intervals (in brackets) Calculated by the Pooled Replicates Method**

Temperature range (°C)	Time (h)	No. of data	Final % degraded	Ea (Kcal/mol)	$t_{90\%}$ (days)	%[a]difference
40–82	168	45	6.2%	24.1[23.6–24.5]	42.2 [38.4–45.2]	–8.21%
40–70	120	33	44%	23.3 [22.5–24.1]	36.9 [30.9–42.8]	+5.38%
40–70	60	33	41%	22.3 [21.4–23.2]	12.8 [10.9–14.7]	+67.2%

[a]Percent difference for the $t_{90\%}$ = (Isothermal-Nonisothermal)/Isothermal·100.

situation in which the Ea and A parameters for two given experiments could differ to a great extent, while the $t_{90\%}$ estimates of the same two experiments could be very similar. In such cases, the selection of the appropriate experimental conditions would be difficult. In principle, this predicament can be considered as a specific problem of nonisothermal studies, and not as a problem of the data-fitting method.

CONCLUSIONS

The results of our study demonstrate that the nonisothermal method provides values of activation energy that are very similar to the values obtained by the isothermal approach, with an error below 2%. The estimated shelf life was also reasonable, but it was significantly higher than the estimated shelf life obtained from isothermal data; an error of 8.2% was detected.

The integral approach is a reliable method for analyzing nonisothermal kinetic data which allows kinetic parameters to be used for predicting peptide stability and thus estimating shelf life. However, it should be noted that the accuracy and precision of estimates could be affected by experimental conditions. Poorly designed studies with short experimental periods and narrow temperature ranges could lead to large experimental errors.

The data presented in this work apply only to the thermal stability of the CCK-8 peptide in aqueous solution at pH 12 and ionic strength of 0.01M. Any extension of the kinetic parameters to other experimental conditions or settings under the influence of radiation (for example, after labelling with technetium) is invalid and such experimental conditions should be further investigated.

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