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An improved methodology for data analysis in accelerated stability studies of peptide drugs: Practical considerations

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ABSTRACT

Although the basic science behind current methods for studying biopharmaceutical drug stability has not changed significantly, the techniques available for predicting stability have evolved over the years. This paper therefore describes and discusses various options of data analysis for accelerated degradation studies of peptide and protein drugs based on the Arrhenius equation. Both linear and non-linear regression analyses are also discussed. The results indicate that the simultaneous treatment of all data, as opposed to determining individual rate constants is clearly preferable, combined with the use of the reparameterized Arrhenius equation. The estimated shelf-life at 5 °C varied between 2.2 and 4.0 years in function of the temperature range and procedure used, whereas the precision of the estimated parameter is reflected in the width of the 95% confidence intervals, the classic Arrhenius analysis was maxima. All these results were evaluated by the bootstrap approach.

1. Introduction

The International Conference on Harmonization (ICH) Guidelines Q5C for testing of biotechnological products advise that the drug manufacturer should provide data on the stability of biopharmaceutical drugs, including the many external conditions that can affect their potency, purity and quality [1]. It is therefore necessary to study the inherent stability of this type of product and identify the problems likely to be encountered in developing a stable formulation, although the time required for these studies at ambient temperature can be lengthy because chemical reactions proceed relatively slowly at low temperatures. Undoubtedly, accelerated and stress stability testing can help determine the most suitable excipients and concentrations [2,3], allowing for a significant reduction in testing time. The Tripartite Guideline on stability testing describes the storage conditions for accelerated and stress studies [4]; a validated stability-indicating method is often required to meet the strict standards set by the regulatory authorities [1]. It is important that these methods be effective enough to predict even slow rates of degradation product formation. Although a variety of analytical methods have been used to characterize the physical and chemical stability of peptides and proteins [5], continuous data evaluation is crucial for the development of stable formulations, since failure can be due to lack of efficacy or an initially poor formulation, but sometimes the end results are unsatisfactory due to inappropriate experimental design and data evaluation.

The recommendations in the evaluation and statistical analysis of stability data provided in the Tripartite Guideline are brief in nature and limited in scope. For example, this guidance states that regression analysis is an appropriate approach to evaluating the stability data for a quantitative attribute (e.g., assay as percent of label claim) and establishing a shelf-life. The relationship can be represented by a linear or nonlinear function on an arithmetic or logarithmic scale. In some cases, a nonlinear regression can better reflect the true relationship, but this guidance do not cover situations where multiple factors are involved in a full-or reduced-design study or do not indicate when and how extrapolation should be performed to calculate the shelf-life. The ICH guidance "*Evaluation of Stability Data* Q1E" expands and analyses these situations, including a decision tree for data evaluation for shelf-life estimation for drugs substances or products (excluding frozen products) [6].

Therefore the purpose of this paper is to review and evaluate various data analysis methods for the stress and accelerated studies of drugs based on the Arrhenius equation in several ways. First, the "classic" and "modified" procedures by linear regression were used. Second, approach by non-linear regression and by the reparameterized Arrhenius equation. In this latter case, the different approaches used to determine the reference temperature were used. In this situation, the Monte Carlo method was used to obtain information about uncertainties in experimental data. All these aspects were analysed experimentally using the stability data of the cholecystokinin fragment CCK-4 in aqueous solution, and are discussed.

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2. Materials and methods

2.1. Materials

The cholecystokinin fragment 30–33 amide (CCK-4, Trp-Met-Asp-Phe-NH_2) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Trifluoroacetic acid (TFA: peptide synthesis grade) and acetonitrile (HPLC grade) were from Merck (Darmstadt, Germany). Deionized water was purified in a MilliQ plus system from Millipore (Molsheim, France).

2.2. RP-HPLC method

The chromatographic system used was a Waters apparatus (Milford, MA, USA) consisting of a pump (600E Multisolvent Delivery System), an auto sampler (700 Wisp Model) and a UV–Vis detector (2487 programmable multi-wavelength model). Elution was performed at room temperature in a Nova pack C-18 column (150 mm × 2.9 mm, 60 Å, 4 μm particle size, Waters). The data was collected and analysed using the Millennium32® chromatography program (Waters).

The mobile phase was an acetonitrile-water (30:70, v/v) mixture with 0.05% TFA, the flow rate 1.0 mL min^{-1}, and injection volume 25 μL. The detection wavelength was set at 280 nm. All solvents were filtered with 0.45 μm (pore size) filters (Millipore) and degassed.

2.2.1. Validation of the RP-HPLC method

Validation was carried out as per the ICH Q2-(R1) guidelines [7], for selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision and robustness.

The results obtained indicate that the method is specific, linear over a concentrations range 2–12 μg mL^{-1}, accurate (recovery mean = 100.2 ± 2.02%), precise (repeatability = 0.67%), and reliable (inter-assay precision = 2.74 0%). The LOD was calculated by statistical methods using a ratio of $3\sigma/s$ (σ: the standard deviation of response; s: slope of the calibration curve). The LOQ was also calculated with a ratio of $10\sigma/s$. The LOD was established at 0.35 μg mL^{-1} and LOQ at 1.06 μg mL^{-1}. Acceptable robustness was also observed, indicating that the analytical method remains unaffected by small but deliberate variations in mobile phase composition and flow rate, as described in the ICH Q2-(R1) guidelines [7].

During preliminary method development work, we tested samples obtained from stability studies with CCK-4. The results obtained from these samples clearly demonstrated that the method was capable of distinguishing the CCK-4 peak from all degradation products in the samples (see Fig. 1), with a good resolution between the peaks II and IV (Rs > 2.7), although a lower value was obtained for the peaks I and III (Rs = 1.25), and the selectivity (α) was always higher than 1 [8].

2.3. Liquid chromatography–mass spectrometry (LC–MS) system

The samples were analysed using an Agilent 1100 (Agilent Technologies, Waldbronn, Germany) LC system interfaced with a Bruker Daltonics micrOTOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an orthogonal electrospray (ESI) ion source. Ionization was performed in the positive mode and ionization parameters were previously optimized. The analysis conditions were identical to those described in the above section, but with a flow rate of 0.7 mL min^{-1}. The analysis run time was 13 min, and the injection volume, 10 μL. Hyphenation Star versions 3.1 by Bruker Daltonics (Bremen, Germany) was used to control chromatography. Mass spectra were collected from m/z 250 to 1500 and processed with Data Analysis 3.3 software. All reported masses are monoisotopic $[M+H]^+$ unless otherwise noted.

2.4. Stability studies

The oven (BR-UT 6000 Model; Heraeus Instruments, Germany) temperature was pre-set and maintained at the desired temperature for accelerated studies. 10 mg of CCK-4 was dissolved in 1 mL of dimethyl sulphoxide (DMSO), and transferred to a 10 mL volumetric flask, immediately followed by addition of NaOH solution (0.01 M) to obtain a final concentration of 1 mg mL^{-1}, and the final pH was adjusted to 12 ± 0.1 [9]. Aliquots of this bulk solution were stored at different temperatures, 40, 50 and 60 °C with variations less than ±1 °C; and also at room temperature protected from light, and thermostatically controlled over six months, the mean temperature being 25.7 ± 0.6 °C. Aliquots were removed from the oven at various time intervals, diluted with the mobile phase to obtain concentration values within the calibration range and analysed the same day in triplicate.

3. Results and discussion

3.1. Degradation products identities and mechanism

The degradation of CCK-4 in solution yielded three peaks: II, III and IV as depicted in Fig. 1. Further examination of the MS spectrum obtained by the LC–MS system indicates that the peak II corresponds to a degradation product with an m/z ratio of 1157.47, i.e. the cyclic dimer. Given the experimental conditions, the formation of cyclic dimer implies two peptide bonds between the two free carboxylic groups of the aspartic residue with the free secondary-amine groups of the tryptophan residue, involving the loss of two water molecules.

The peaks III and IV correspond to degradation products with m/z ratios of 993.38 and 845.31, respectively, which derived from the cyclic dimer. Thus, the peak III correspond to the loss of a phenylalanine-amide residue (Phe-NH_2), whereas the peak IV correspond to the loss of two Phe-NH_2 residues after addition of a hydroxyl group ($\Delta m/z$ = +17). This result is consistent with a cleavage reaction on the C-terminal side of the aspartic acid residue, this bond being particularly liable to hydrolytic cleavage. Cleavage on the $n-1$ and $n+1$ side (i.e. N- and C-terminal sides) of aspartic acid involves the formation of an anhydride intermediate [10]. Joshi and Kirsch has proposed that the $n-1$

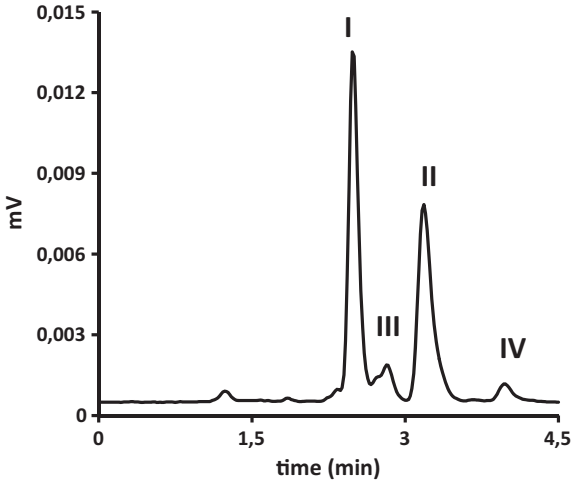

Fig. 1. Chromatogram of a CCK-4 sample stored at 60 °C for 5 days, showing the three degradation products, identified as cyclic dimer (II), whereas peaks III and IV derived from the cyclic dimer after the loss of one (III) or two Phe-NH_2 (IV) residues.

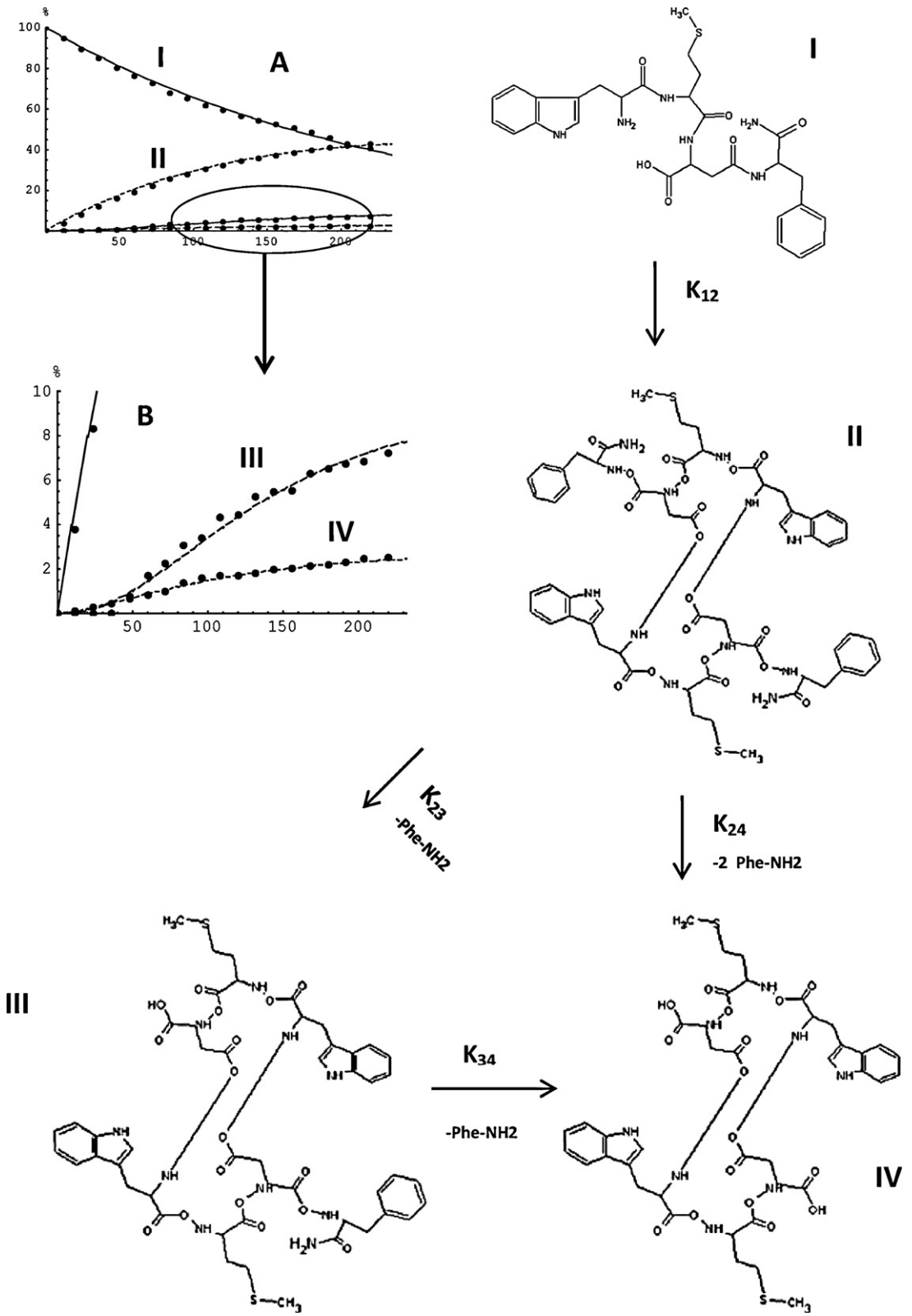

Fig. 2. Degradation scheme proposed for the CCK-4 loss and degradation product appearance and its loss. Variation of the CCK-4 loss and cyclic dimer formation (A) follow a first-order kinetic, and (B) formation of two products derived from the cyclic dimer fit a consecutive reaction.

cleavage reaction is slower than $n+1$ cleavage because the formation proceeds via a six-member ring intermediate rather than via a five-member ring [10], and second, the $n+1$ cleavage would always precede $n-1$ cleavage, although this process can occur consecutively and in parallel. In the study described here, no peak implying $n-1$ cleavage was detected.

Peak IV could either be formed from peak II or via peak III following a consecutive reaction, although the possibility of both

consecutive and parallel pathways cannot be ruled out. To investigate this, two models (see Fig. 2) were evaluated and compared to determine the relative importance of different degradation pathways for the cyclic dimer.

The rate constants k_{12}, k_{23}, k_{24} and k_{34} were estimated by simultaneously fitting the concentration-time profiles for CCK-4 loss and degradation product appearance and loss using models as depicted in Fig. 2. For this, the *NonLinearFit* function from Mathematica® program [11] was used to resolve the equations differentials for curves fitted.

The results obtained in the assay at 60 °C shows that the cyclic dimer degradation follows both consecutive and parallel process where the rate constant for the step II → IV was 3.5 times lower than for the step II → III ($k_{24} = 8.55 \times 10^{-4}$ vs $k_{23} = 2.99 \times 10^{-3}$ h^{-1}), and the 95% confidence intervals for these estimated parameters does not include the value 0, which indicate that these parameters are significant ($p < 0.05$) and therefore this process cannot be disregarded.

However, when the data fit a consecutive reaction or both consecutive and parallel process do not differ, the residual sum of squares (RSS) is slightly higher for both process (42.1 vs 40.02), but the increased number of parameter does not produce a significant improvement in the fit. In this situation, the *F*-test can be used to discriminate between models that are hierarchical [12]. The *F*-test results suggest that the model with both process offers not significant improvement over the model with the consecutive reaction, since the calculated value ($F = 3.69$) is lower than the tabled value ($F = 3.98$). Thus, the simpler model should be adopted, i.e. the consecutive reaction. This behaviour was also observed for the rest of storage conditions, the ratio between the rate constants (k_{23}/k_{34}) being very similar (data not published).

These results indicate than the main pathway for CCK-4 degradation is the dimerization reaction followed by the hydrolytic cleavage of Phe-NH_2 residue (see Fig. 2). The first step follows a first-order reaction, whereas the second is a first-order consecutive reaction.

3.2. Data analysis using the Arrhenius equation by linear regression

The suitability of the Arrhenius relationship to different chemical degradation pathways of peptides and proteins, such as deamidation, hydrolysis, racemization, and polymerization, has been studied by several authors [10,12–19]. However, one of the main problems found was to determine a suitable temperature range for stability studies, where the degradation mechanism is the same and, so that the Arrhenius plots would then be valid.

At first, the storage conditions corresponding to the general case described in the ICH-Q1A-(R2) were used [4], but when accelerated data show change over time, testing at the intermediate condition (e.g. 25 ± 2 °C) should be performed. The Arrhenius plot shown in Fig. 3 indicates that the degradation mechanism and kinetics do not change with temperature. The activation energy was 26.6 kcal mol^{-1}, higher than those found for similar peptides [18], although this result is not strange since the degradation mechanisms are different. To calculate the shelf-life ($t_{90\%}$), it is previously necessary to estimate the rate constant at ambient temperature (25 °C), obtaining a value of 44.3 days, whereas the experimental value was 36 days, the error being of 23%. Estimating this according to the regression equation derived from the Arrhenius plot resulted in a calculated degradation rate that deviated significantly from that observed experimentally. In classical Arrhenius analysis, the rate constants are calculated by fitting normal degradation vs time data, and then fitting these calculated rate constants back to the Arrhenius equation. The errors included in the original data points are thus not directly reflected in the Arrhenius plots, and the weight

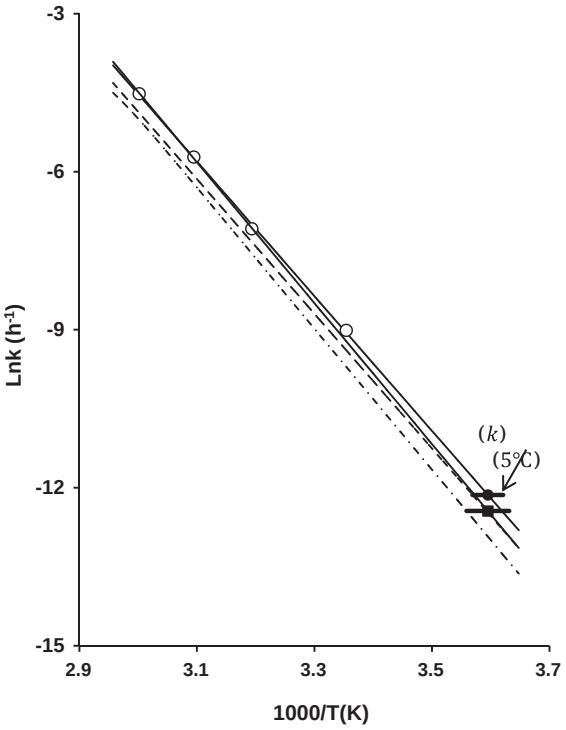

Fig. 3. Plot obtained by classical Arrhenius analysis using 3 or 4 temperatures. Dashed curves represent 95%-confidence intervals and dark lines represent the length of 95%-confidence intervals for estimated rate constant at 5 °C for each temperature range.

given in the analysis to a single data point varies if the number of data points differs among the different temperature levels. The variations in the rate constant estimated from the Arrhenius plot become greater owing to the reduction in the degrees of freedom. This occurs because there are less calculated rate constants than the original points used to estimate the original experimental rate constants [20].

The ICH Guideline Q1A [4] states that when accelerated data show change over time, testing at the intermediate condition (e.g. 25 ± 2 °C) should be taken into consideration, whereas the $t_{90\%}$ estimation should performed for storage below room temperature, i.e., 5 °C mean values corresponding to the interval recommended for this type of products (2–8 °C). In this situation, the *Ea* value was slightly lower, 25.4 kcal mol^{-1}, due to a slight change in the slope (Fig. 3), whereas the $t_{90\%}$ estimated for 5 °C was 2.25 years, lower than the estimated value using three temperatures, 3.05 years. These results highlight the need to include temperatures close to those recommended for storage in order to avoid underestimating shelf-life, in our case by 35.5%.

However, not only the kinetic parameters themselves but also the confidence in their values is important for interpreting the differences, especially regarding the $t_{90\%}$. The uncertainties given as 95% confidence intervals were calculated from the residual standard deviation by the standard expression [21]. The uncertainties are quite large due to the few degrees of freedom based only on the scatter in the Arrhenius plot, and even larger for the temperature range from 40 to 60 °C ($n = 3$). For example, the estimated $t_{90\%}$ was 3.05 years with 95% confidence intervals of 0.61–15.1 years, whereas for the temperature range from 25 to 60 °C ($n = 4$), the $t_{90\%}$ was 2.25 years with a 95% confidence interval from 1.23 to 4.09 years. These calculations were made on the assumption that the activation energy remains constant over the temperature range 5–60 °C. The confidence intervals are very large, since there is a coupling of the uncertainty of the prediction due to fitting the rate

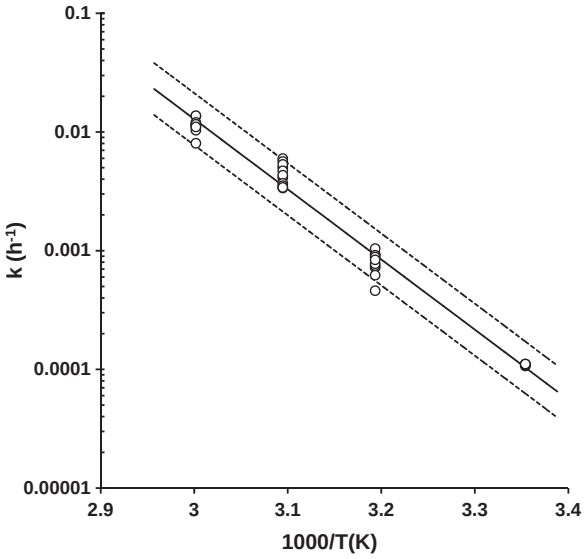

Fig. 4. Arrhenius plot obtained by the "modified" analysis. The upper and lower curves represent 95%-confidence intervals.

constants at each temperature with the temperature dependence due to extrapolation to storage temperature. This effect is greater in the shorter temperature range, since the number of temperatures and the amplitude of their range are smaller, and the extrapolation is performed too far from the mean temperature within the experimental temperature range (50 vs 41 °C).

The Arrhenius regression analysis can also be performed using the multiple rate constants calculated from each of the drug-concentration/time data points observed at a single temperature level [20]. As shown in Fig. 4, the data at various temperature levels are fitted to Eq. (1) for first-order degradation kinetics, obtained by replacing the rate constant k of the Arrhenius equation with the remaining drug concentration, [D]:

$$\ln\frac{[D]}{[D_0]} = -tk_0 \exp\left(\frac{-Ea}{RT}\right) \quad (1)$$

where [D_0] is initial drug concentration. This "modified Arrhenius analysis" using weights each data equally and provides an estimate of the rate constant at room temperature with a smaller 95% confidence interval, owing to the larger number of data points used. This approach was applied to the CCK-4 data in order to compare it with the classical Arrhenius analysis. The results are summarized in Table 1. Nevertheless, this method provided a regression curve and calculated room-temperature degradation rate similar to those observed in the classical Arrhenius analysis, but with the additional advantage that the calculated uncertainties in the estimated parameters reflect the real scatter in the experimental data (see Fig. 4). The estimated shelf-life was close to that derived from linear regression analysis; a difference of 4.2 days was observed, but significantly more accurate, as seen in the length of the 95% confidence interval for the estimated parameter (Table 1). This is due to the increased degrees of freedom in the modified Arrhenius analysis, although the difference in the estimated variances was highest (s^2 = 0.0706 vs s^2 = 0.00107 for the classical analysis) since the dispersion in the individual observations was higher. Further examination of the residuals revealed that each of the basic regression assumptions (normality, constant variance, and independence of observations) were correct.

If the ambient temperature assay was included, the difference between the estimated $t_{90\%}$ at 5 °C was greater than six months, but the confidence intervals were also narrower (see Table 1). However, the results obtained are close to those obtained by the classic analysis in the 40–60 °C temperature range; because the dispersion of the data at lower temperatures is lower in comparison with higher temperatures which cause a change in the slope.

3.3. Data analysis of the Arrhenius equation by non-linear regression

Although linear regression analysis provides biased results and weighted least-squares analysis is required to improve the estimates, non-linear regression analysis does not suffer from the same problem [20]. To analyses this, the Arrhenius parameters were calculated directly by non-linear regression for both temperature ranges. The results are summarized in Table 1. In this study, the *Nonlinear Fit* function from the Mathematica® program was used [11]. The maximum number of iterations in the search was 30 and the χ^2 function was minimized with the Levenberg–Marquardt method. The initial estimated values for the Arrhenius parameter, obtained from the classic analysis, were used as starting values in order to converge much faster to a solution. An optimal value of 25.9 kcal mol^{-1} for the activation energy and 2.55 years for the $t_{90\%}$ at 5 °C were obtained, slightly lower than those obtained by linear regression, whereas the 95% confidence intervals shown in Table 1 were estimated by the asymptotic standard errors method.

Yoshioka and Stella [20] propose the non-linear representation Eq. (1) yields Eq. (2) for first-order degradation kinetics. The frequency factor k_0 corresponds to the rate constant at infinite temperature.

$$[D] = [D_0]\exp\left[-tk_0 \exp\left(\frac{-Ea}{RT}\right)\right] \quad (2)$$

As an alternative to Eq. (2), these authors replace the parameter k_0 with k_{298}, which has a more practical meaning than k_0 [rate constant of degradation at 25 °C represented by Eq. (3)], yielding Eq. (4):

$$k_0 = k_{298}\ \exp\left(\frac{Ea}{298R}\right) \quad (3)$$

$$[D] = [D_0]\exp\left\{-k_{298}t \exp\left[\frac{Ea}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right]\right\} \quad (4)$$

Again, replacing k_{298} in Eq. (4) with $t_{90\%}$, yields Eq. (5)

$$[D] = [D_0]\exp\left\{-\frac{0.1054 \cdot t}{t_{90\%}} \exp\left[\frac{Ea}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right]\right\} \quad (5)$$

The estimates of $t_{90\%}$ and Ea can be obtained directly by non-linear regression analysis of the observed degradation vs time data according to Eq. (5). This is their main advantage with respect to Eq. (2), since it is necessary to previously estimate the rate constant at 25 °C, and thus $t_{90\%}$. For this, the data were analysed by non-linear regression using Eq. (5), but $t_{90\%}$ was also calculated at 5 °C. The Ea and $t_{90\%}$ values were quite similar to those obtained by linear regression, but the 95% confidence intervals were smaller (Table 1), demonstrating that more precise estimates could be obtained using all the data instead of determining individual rate constants.

The estimation of confidence intervals in nonlinear models is not as straightforward as with the linear models, they are not symmetric and can be underestimated [22]; the extent of the error depends on the nonlinearity of the model and the number of data. There are various methods to evaluate confidence intervals, such as minimization of the χ^2 functions or F-distribution, but the best option is via the Monte Carlo method, which is a practical means of quantifying the risk associated with uncertainty in process parameters [23]. In a Monte Carlo simulation, uncertain input variables are represented by probability distributions. A simulation calculates numerous scenarios of a model by repeatedly picking values from a user-defined probability distribution for the uncertain variables. It then uses those values in the model to calculate and analyses

Table 1
Arrhenius parameters and shelf-life with their 95%-confidence intervals (in brackets) obtained by non-linear and linear regression methods according to the temperature range.

Parameter	Classical	Modified	RNL[a]	Yoshioka and Stella	Bootstrap[b]
Temperature range: 40–60 °C					
Ln A (days^{-1})	35.62 [26.11–45.13]	37.99 [33.95–42.03]	34.64 [26.93–42.35]	36.18 [34.25–38.12]	36.19 [34.49–37.89]
Ea (kcal mol^{-1})	26.56 [20.46–32.66]	28.02 [25.43–30.61]	25.92 [20.84–31.01]	26.90 [25.66–28.14]	26.90 [25.80–28.00]
$t_{90\%}$ (25 °C), days	44.3 [17.6–111.6]	48.5 [14.5–162.7]	40.1 [16.6–96.5]	44.3 [36.8–51.8]	44.4 [38.14–51.30]
$t_{90\%}$ (5 °C), years	3.05 [0.61–15.1]	3.99 [0.49–32.5]	2.55 [0.57–11.4]	3.17 [2.17–4.18]	3.21 [2.41–4.18]
Number of data	3	30	3	35	10,000
Temperature range: 25–60 °C					
Ln A (days^{-1})	33.82 [30.20–37.45]	36.50 [34.28–38.72]	34.63 [32.76–36.49]	35.43 [34.32–36.54]	35.46 [34.45–36.46]
Ea (kcal mol^{-1})	25.40 [23.12–27.68]	27.06 [25.66–28.46]	25.91 [24.68–27.14]	26.41 [25.71–27.11]	26.43 [25.79–27.07]
$t_{90\%}$ (5 °C), years	2.25 [1.23–4.09]	3.10 [1.14–8.42]	2.54 [1.77–3.65]	2.79 [2.33–3.25]	2.81 [2.41–3.25]
Number of data	4	36	4	43	10,000

[a] RNL: non-linear regression.
[b] Estimated value with 95% confidence intervals based on bias-corrected and accelerated (BCA) bootstrap method.

the outputs in a statistical way in order to quantify risk. The outcome of this analysis is an estimation of the confidence with which the desired values of key performance indicators can be achieved. The Monte Carlo approach was used for deriving confidence intervals for $t_{90\%}$ and Ea, and confirms that their distributions are also normal. In this case, the Mathematica® program was used [11]; 10.000 bootstrap samples were performed on these parameters. The asymptotic and bootstrap confidence intervals were very similar. Also, this approach gave closely similar uncertainty estimates to those obtained using both linear and non-linear analysis. Overlapping of 95% confidence intervals indicates that the parameters derived by the two procedures do not differ (Table 1). Also, to check if the normality of the empirical bootstrap distributions for both parameters is appropriate, their normal quantile–quantile plots were analysed. From these plots, it can be deduced that the assumption of normality is correct, since the points lie roughly on a straight line (Fig. 5).

3.4. Reparameterization of the Arrhenius equation

The use of the so-called linear form of the Arrhenius equation is controversial [24–26]. Schwaab and Pinto [26] hold it should

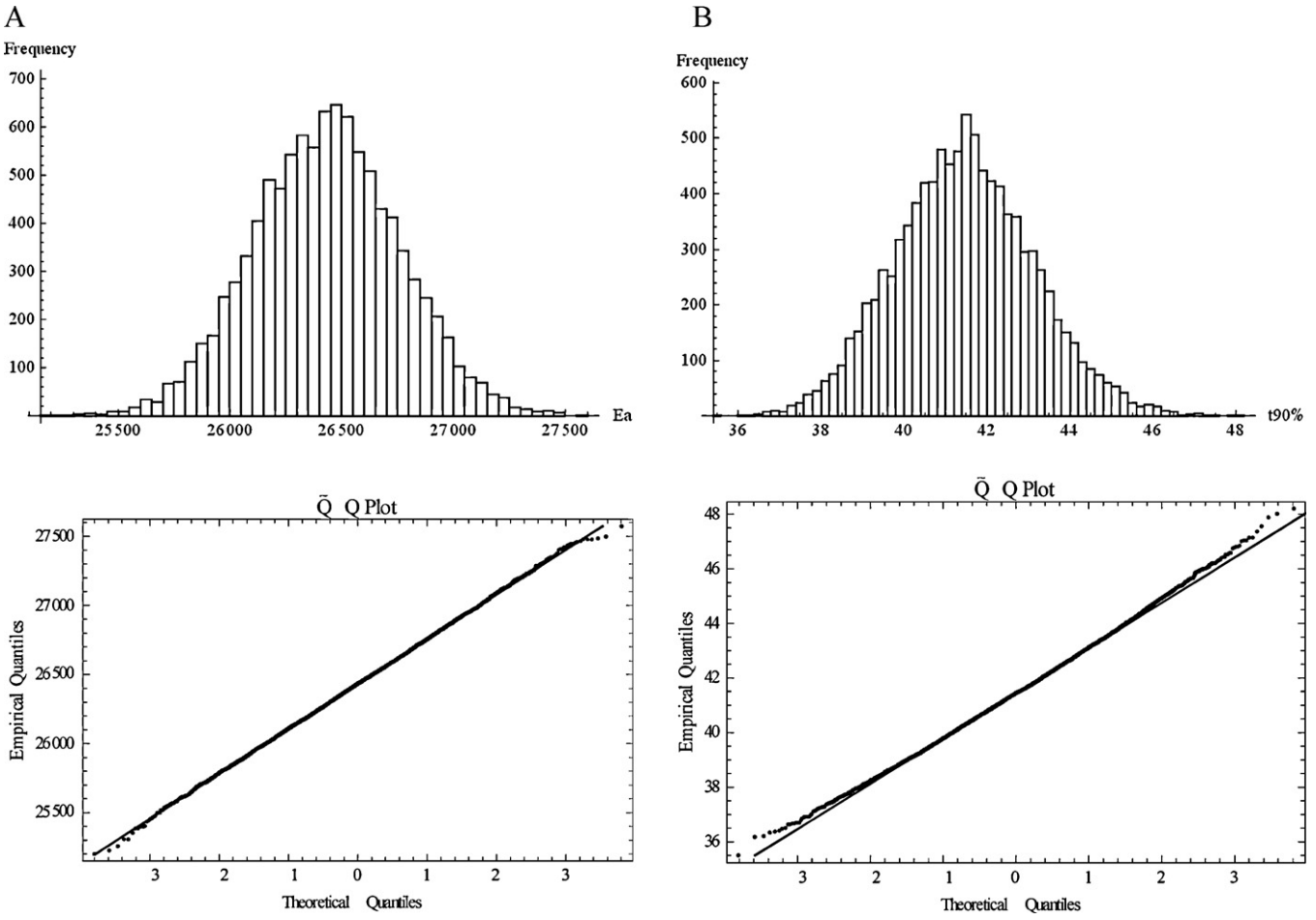

Fig. 5. Histograms of 10,000 bootstrap iterations and the quantile of standard normal plots for activation energy (A) and shelf-life (B) parameters estimated by the Yoshioka and Stella approach.

be avoided and the parameter estimation procedure must preserve statistical meaning, leaving explicit the error structure of the experimental observations. These authors also recommend inserting the Arrhenius equation into the kinetic rate expression and that all parameters are estimated simultaneously using the complete set of available experimental data. The high correlation between parameter estimates may derive from several sources, such as an inappropriate model, bad experimental design and/or the non-linearities in the model. This is exactly the case of the Arrhenius equation, since exponentiating the reciprocal of the absolute temperature introduces a high correlation between the frequency factor and the activation energy. In order to minimize this correlation, one may reparameterize the Arrhenius equation in one of the following suggested forms [27] with a definition of a reference temperature, T_{ref}.

$$k = \exp\left[B - \frac{Ea}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \quad (6)$$

where the parameters of the reparameterized equations can be related to the parameters of the traditional Arrhenius equation in the form:

$$B = \ln(k_{T_{ref}}) = \ln(k_0) - \frac{Ea}{RT_{ref}} \quad (7)$$

It should be observed that $k_{T_{ref}}$ is the specific reaction rate at the reference temperature T_{ref}. Besides, if T_{ref} is infinite, Eq. (6) becomes equal to the Arrhenius equation. Therefore, the frequency factor k_0 of the original Arrhenius equation can be understood as the specific reaction rate at infinite temperature. The parameters B and $k_{T_{ref}}$ depend on the definition of the reference temperature.

All proposed reparameterized forms of the Arrhenius equation thus depend on a new parameter, T_{ref}. Little attention has been given in the literature to its value, which is commonly defined as the average temperature of the analysed experimental range. For instance, Veglio et al. [28] suggested the use of the inverse average temperature

$$\frac{1}{T_{ref}} = \frac{1}{NE}\sum_{i=1}^{NE}\frac{1}{T_i} \quad (8)$$

where NE is the number of experimental temperature values and T_i is the temperature for individual experiments. According to Schwaab and Pinto [27], the optimum reference temperature is the reciprocal of the weighted average of the T_i values used in the experiments, and the weights depend on the experimental values.

To illustrate the high parameter correlation and how it can be diminished through reparameterization of the Arrhenius equation, the data sets of CCK-4 were analysed. Assuming a first order kinetic model, the remaining fraction "C_i" can be expressed as:

$$C_i = \exp\left\{-t_i k_{T_{ref}} \exp\left[-\frac{Ea}{R}\left(\frac{1}{T_i} - \frac{1}{T_{ref}}\right)\right]\right\} \quad (9)$$

where the reparameterized form defined in Eq. (6) was used. In this situation, the optimum reference temperature is:

$$T_{ref} = \frac{\sum_{i=1}^{NE}[C_i \ln(C_i)]^2}{\sum_{i=1}^{NE}[C_i \ln(C_i)]^2/T_i} \quad (10)$$

The kinetic parameters were estimated by non-linear regression, using a reference temperature of 318.07 K, calculated according to Veglio et al. [28], where the temperature range between 25 and 60 °C was considered. As expected, the performance of the model is not affected by the reparameterization. The Ea value is the same, 26.41 kcal mol^{-1}, whereas B depends on the reference temperature used but the frequency factor is the same, an average value of 35.43 (as logarithm) was obtained. The $t_{90\%}$ calculated at 5 °C was 2.79 years, close to those derived from the classical Arrhenius approach, but significantly more accurate, as can be seen in the length of the 95% confidence intervals (see Table 1). For this reference temperature, the correlation parameter becomes −0.0239969, a value 40 times lower than with the traditional form of the Arrhenius equation (−0.999995), whereas the T_{ref} obtained with Eq. (10) becomes 318.09 K, which leads to a similar parameter correlation. The small difference observed between the two references Temperature is due to using experimental values of C_i in Eq. (10), whereas if the estimated values are used, the T_{ref} is the same as those obtained by Veglio et al. [28].

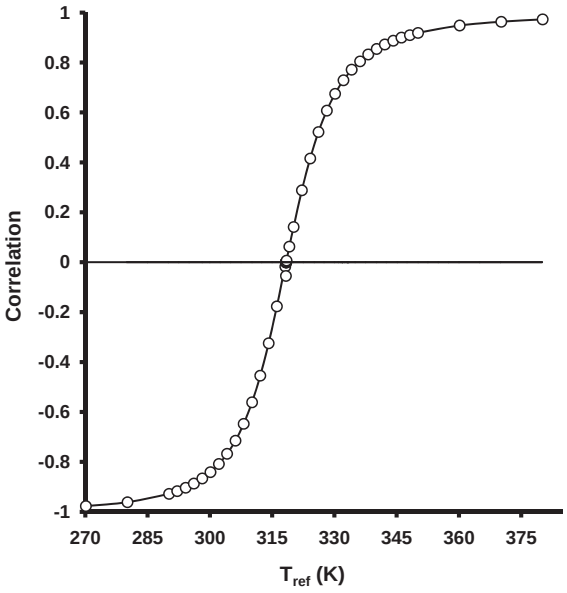

Fig. 6. Effect of the reference temperature on the Arrhenius parameter correlation.

If the reference temperature of 316.9 K is used, i.e. the midpoint temperature value within the analysed experimental range, the parameter correlation becomes −0.11784. This is worse than those obtained with either of the two approaches described earlier. In order to evaluate the effect of the reference temperature on the parameter correlations, it was allowed to vary (Fig. 6) where the parameter correlation is close to −1.0 for T_{ref} values lower than the optimum, and approaches the value of +1.0 when higher. In this case, the minimum correlation parameter (0.00007616) was obtained at T_{ref} = 318.37 K, very far from the midpoint temperature, but close to those calculated in accordance with Veglio et al. [28]. This difference between the two reference temperatures is because the weight given in the calculation varies according to the data points between the different temperature levels. This becomes greater at higher temperatures, resulting in a larger contribution.

3.5. Uncertainty of rate constant k

All models and equations that are derived originally from experimental data have a degree of uncertainty associated with their calculations or predictions, but they are often used without taking such uncertainty into account. However, experienced practitioners will always allow a safety factor to compensate for a margin of error, even if the size of that error is not unknown. When the original data and the model are both available, statistical methods can be used to quantify the uncertainty level. This makes the potential deficiencies of the model more evident and explicit and may also focus further experimentation on reducing those uncertainties. More details about how to calculate uncertainty are given elsewhere [29–31].

Table 2
Uncertainty of shelf-life ($t_{90\%}$) estimated by the approaches assessed.

Procedure	s^2 (Ea)	s^2 ($\ln k_0$)	Covariance	$t_{90\%}$ (years)	Uncertainty[a]
Classical Arrhenius	70,810	0.7087	−223.8	2.25	0.86–5.88
Modified Arrhenius	120,530	1.192	−378.7	3.10	0.88–10.89
Non-linear regression	81,655	0.187	123.7	2.55	0.91–7.16
Yoshioka and Stella	120,613	0.302	190.7	2.79	0.79–9.80
Reparameterized Arrhenius	s^2 (Ea)	s^2 (B)	Covariance	$t_{90\%}$ (years)	Uncertainty[a]
T_{ref} = 316.9 K	120,613	4.66×10^{-4}	−0.8832	2.79	0.61–12.83
T_{ref} = 318.07 K	120,613	4.60×10^{-4}	−0.1787	2.79	0.90–8.61
T_{ref} = 318.09 K	120,613	4.60×10^{-4}	−0.1667	2.79	0.91–8.55
T_{ref} = 318.37 K	120,613	4.59×10^{-4}	0.0005669	2.88	1.02–8.14

[a] Expanded uncertainty with a coverage factor "K" of 2.

In general terms, the evaluation of uncertainty is based on estimating the standard deviation associated with all the sources of variability that affect the measurement process. Standard uncertainty ($u(x)$) can be expressed as a standard deviation, and expanded uncertainty ($U(x)$) is calculated by combining standard uncertainty with a coverage factor K; a value of 2 is usually chosen to obtain a confidence level of 95% [30]. In some cases, it is feasible to use relative uncertainty (in both uncertainties), which represents a normalized uncertainty value as the quotient between the standard uncertainty $u(x)$ and x itself.

With the parameters estimated from the Arrhenius equation, k-values can be predicted at every temperature from the Arrhenius equation, while the uncertainty in predicted k-values can be calculated from the theory of error propagation [29,32].

$$\sigma_k^2 = \left(\frac{\partial k}{\partial k_0}\right)^2 \sigma_{k_0}^2 + \left(\frac{\partial k}{\partial Ea}\right)^2 \sigma_{Ea}^2 + 2\left(\frac{\partial k}{\partial k_0}\right)\left(\frac{\partial k}{\partial Ea}\right)\sigma_{k_0 Ea} \quad (11)$$

which results in:

$$\left(\frac{s_k}{k}\right)^2 = \frac{s_{k_0}^2}{k_0^2} + \frac{s_{Ea}^2}{(RT)^2} - \frac{2}{k_0 \cdot RT} s_{k_0 Ea} \quad (12)$$

If the reparameterized Arrhenius Eq. (6) is used:

$$\left(\frac{s_k}{k}\right)^2 = \frac{s_{k_0}^2}{k_{T_{ref}}^2} + \frac{((1/T) - (1/T_{ref}))^2 s_{Ea}^2}{R^2} - \frac{2((1/T) - (1/T_{ref}))s_{k_0,Ea}}{R \cdot k_{T_{ref}}} \quad (13)$$

where $s_{k_0}^2$ y s_{Ea}^2 are the variances for the activation energy and pre-exponential factor, respectively, and $s_{k_0,Ea}$ is the covariance between them. The Mathematica® program [11] was used to estimate the different parameters and the matrix of variance-covariance of each approach. The estimated rate constant at 5 °C did not differ more than 1%, although the uncertainty depends on the method applied (Table 2).

In principle, the factor most influencing the combined uncertainty should be the covariance of the parameters. Thus, the uncertainties were higher for the "modified" Arrhenius procedures since the covariance obtained was negative and higher, whereas for the non-linear regression approaches, although the covariances were positive, their contribution was not enough to diminish the effect of the variance of the activation energy, the most influential factor. This fact is also shown in the classic Arrhenius approach, but in this specific case, the variance of the activation energy is lower and is able to reduce the effect of the negative covariance, and thus, the uncertainty is the lower.

However, for the reparameterized Arrhenius expression the main factor is the covariance of the parameters, because the variance of the activation energy is the same; whereas the contribution due to the frequency factor is very small. In this case, the lowest uncertainty was obtained for a T_{ref} of 318.37 K, since the covariance was positive and very near zero (Table 2), whereas for 316.9 K, the uncertainty in k was higher since the covariance was negative and close to one (−0.883253). This fact is reflected in the width of the 95% confidence intervals (Table 2).

4. Conclusions

The stability data sets of CCK-4 in solution were analysed using both non-linear and linear regression methods. The least-squares regression analysis is the first option to check the validity of the model and reliability of the estimated parameters, although non-linear regression analysis is nowadays a good alternative, especially since the introduction of computer programs and packages that allow it to be performed very easily. The results highlight the need to include temperatures close to those recommended for this type of products in order to avoid underestimating shelf-life since the estimated value for 5 °C can vary between 2.2 and 4.0 years in function of the temperature range and procedure used. Second, the simultaneous treatment of all data is clearly preferable combined with the use of the reparameterized Arrhenius equation as opposed to determining individual rate constants since the uncertainty obtained was lowest. In this case, the $t_{90\%}$ estimated was 2.8 years.

However, the ICH Guideline Q1E indicates that if significant change occurs between 3 and 6 months' testing at the accelerated condition, the proposed shelf-life should only be based on the long-term data since the extrapolation is not considered appropriate. The preliminary long-term stability data seem to indicate that the $t_{90\%}$ at 5 °C is, at least, six months (data not shown).

The results obtained pointed out that data analysis procedures used allow control the risk of falsely decision and provide enough power to correctly concluding stability. This type of analysis is vital for pharmaceutical industry since the products fulfill the requirements must be controlled and the consumer protected.

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