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Evaluation of non-isothermal methods in stability studies of human insulin pharmaceutical preparations

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ABSTRACT

The purpose of this research was to study the thermal stability of a human insulin pharmaceutical preparation using non-isothermal conditions and comparison with classical isothermal experiments. The isothermal studies were performed in the temperature range 20–60 °C, whereas non-isothermal stability studies were performed using a linear increasing temperature program, heating rate 0.25 °C per hour and temperature interval 30–70 °C.

Under isothermal conditions, an apparent first-order degradation process was observed at all temperatures. The linear Arrhenius plot suggested that the insulin degradation mechanism was the same within the studied temperature range, with quite large uncertainties due to the small number of degrees of freedom based only on the scatter in the plot, giving an estimated shelf-life at 25 °C of 199.1 days. In non-isothermal conditions, the integral approach was used to estimate the activation parameters. It provides results in good agreement with those of the traditional method, but with the advantage that the uncertainty in the final result directly reflects the goodness of fit of the experimental data, since it takes into account the scatter in the original data. The estimated shelf-life in non-isothermal conditions was quite close to the value derived from isothermal data, 191.4 days, although the 95% confidence interval estimated were slightly higher. This is due to the differences in the estimation method and the nature of the experimental errors.

The bootstrap technique is also applied to estimating confidence limits for the Arrhenius parameters and shelf-life. This method is very useful when the underlying distribution function of the parameters is unknown. The results obtained indicate that the Arrhenius parameters follow a normal distribution, whereas the shelf-life follows a log-normal distribution. In any case, the results obtained show that there is no difference between the asymptotic and bootstrap confidence intervals.

1. Introduction

The physicochemical characterization of drugs currently involves stability studies. Spectroscopic and chromatographic methods are being commonly used for establishing the corresponding concentration profiles and obtaining kinetic information. The time required to study drug-degradation kinetics at ambient temperature can be very lengthy because chemical reactions proceed relatively slowly at low temperatures. The accelerated isothermal testing at high temperatures allows for a significant time-saving; however, preparation and assay of the large number of samples produced by the multiple-temperature accelerated test may offset this benefit. In non-isothermal studies, the temperature dependence with time is predetermined and rate constants at different temperatures can be calculated, by estimating kinetic parameters from a single set of drug concentration versus time data. This data is obtained while the temperature is changed during the time period according to some algorithm [1]. However, the complexity of a non-isothermal study primarily arises from the temperature-rise program and the associated data-treatment method. The derivative and integral methods are two possible directions in non-isothermal data analysis [1–8]. The experimental application of these techniques in drug stability studies has been very limited [5,7,9,10]. In the case of peptides and proteins only the stability of short peptides in aqueous solution has been studied. For example, Mu-Lan and Stavchansky [8] used thymopentin and its analogs, a pentapeptide, whereas Oliva et al. [11,12] used two cholecystokinin fragments, CCK4 (a tetrapeptide), and CCK8, an octapeptide.

The accuracy and precision of the estimates obtained by the non-isothermal method depend largely on the experimental conditions, such as experimental period, sampling frequency, temperature rise programs, extent of drug degradation, experimental error, mean

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temperature and amplitude of temperature range. Various real or simulated stability studies have been performed to analyse the above mentioned factors [5,7–14].

However, the confidence in estimate values is important, and, consequently, statistical evaluation needs to be considered. The Monte Carlo method, also called the "bootstrap", a randomized re-sampling technique, provides a versatile and reliable statistical method to ensure the accuracy of parameters calculated from experimental data. This technique may be useful for analysing data sets where prior information is sparse, distribution assumptions are unclear and further data difficult to acquire. It is an empirically based method, in which large numbers of simulated datasets are generated by computer from existing measurements, so that confidence intervals for the derived parameters may be obtained by direct numerical evaluation. This method has no constraint upon the number of times that a datum is represented in a re-sampled subset, whose size can be fixed arbitrarily; it is independent of the experimental design parameters and may even exceed the total number of data samples [15,16].

The aim of this work was to explore the potential of predicting isothermal behaviour from non-isothermal data. Also, the reliability of the statistics for the estimates of shelf-life ($t_{90\%}$), activation energy (Ea) and frequency factor (A) obtained by the integral approach was evaluated by the Monte Carlo method. For this, the stability of a human insulin pharmaceutical preparation, a regular rapid acting solution, was evaluated using both isothermal and non-isothermal assays.

2. Experimental

2.1. Materials and reagents

A commercial human insulin preparation was purchased from Novo Nordisk (Bagsvaerd, Denmark). Acetic acid and acetonitrile (HPLC grade) and trifluoroacetic acid (TFA; peptide grade synthesis) were obtained from Merck (Darmstadt, Germany). L-Arginine was purchased from Sigma Chemical Company (St. Louis, MO, USA). Deionized water was purified in a MilliQ plus system from Millipore (Molsheim, France) prior to use.

2.2. SE-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 UV–vis detector (2487 programmable multi-wavelength model). Elution was performed at room temperature in a Protein Pack column (60 Å, 15 μm, 7.8 mm × 300 mm, Waters). The data collection and analysis were performed using the Millenium32® chromatography program (Waters).

The mobile phase was a mixture (v/v) of 2.5 M acetic acid, 4 mM L-arginine, and acetonitrile (96:4), the flow rate 1.0 ml/min, and the injection volume 50 μl. The detection wavelength was set at 280 nm. All solvents were filtered with 0.45 μm (pore size) filters (Millipore) and degassed.

The SE-HPLC method was validated according to the International Conference on Harmonization (ICH) Guidelines [17]. The results obtained in the validation process indicate that the method is specific, linear over a range of concentrations of 8.75–87.5 μg/ml, accurate (recovery mean = 96.5 ± 3.70%), precise (repeatability = 0.37%), and reliable (inter-assay precision = 3.45%). The limit of detection was established at 3.5 μg/ml and limit of quantitation at 9.45 μg/ml. Acceptable robustness indicates that the analytical method remains unaffected by small but deliberate variations in mobile phase composition and flow rate, which are described in the ICH Q2 (R1) guidelines [17].

2.3. MALDI-TOF mass spectrometry

Mass spectra were measured on an Autoflex MALDI-TOF Mass Spectrometer (Bruker Daltonics, Bremen, Germany). The mass spectrometer was equipped with a 337 nm nitrogen laser, delayed extraction, a reflectron and a hydrophobic coating sample target (Anchor Chip 600/384, Bruker Daltonics, Bremen, Germany). The spectra were obtained using positive ion mode and the accelerating voltage was set to 20 kV. The external calibration of the mass spectrometer was performed with a commercial standard of proteins mixture (Bruker Daltonics) and the standard mass deviation was less than 10 ppm. The mass spectra were accumulated for approximately 500 shots per preparation in total, 30 shots each. All reported masses are monoisotopic $[M+H]^+$ unless otherwise noted.

The matrix used was 2,5-dihydroxybenzoic acid (DHB; Sigma) in a saturated solution (0.16 mg/ml in acetonitrile and 0.1% TFA). Samples were deposited on MALDI targets by spotting 0.5 μl of matrix solution, dried and followed by 0.5 μl of sample.

2.4. Stability studies

2.4.1. Isothermal study

The oven (BR-UT 6000 Model, Heroes Instruments, Germany) temperature was pre-set and maintained at the desired temperature for isothermal studies. The preparation was stored at different temperatures, 40, 50 and 60 °C with variations less than ±1 °C. At the time, the preparation was also stored protected from light at room temperature, thermostatically controlled over 2 years, the mean temperature value being 20.7 ± 1.6 °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 analyzed the same day in triplicate.

2.4.2. Non-isothermal study

The temperature of the oven was controlled by a loop control program written in Test Point® (version 4.0) designed to run from 30 to 90 °C, the total reaction time was 10 days to obtain a linear heating rate of 0.25 °C per hour. Samples were prepared and analyzed as under isothermal studies, but were removed from the oven every 12 h until all data points were collected.

2.5. Non-isothermal studies data analysis

The integral methods was used to analyze the non-isothermal stability data, which are well documented and described in the pharmaceutical literature [5,7–11].

For a first-order reaction, a combination of the integrated rate expression and the Arrhenius equation yields

$$C = C_0 \exp^{-A\int_0^t \exp[-\mathrm{Ea}/RT(t)]dt} \quad (1)$$

This method involves direct evaluation of the integrated expression on the right of Eq. (1). For this, the R® statistical program [18] was used, which allows a direct nonlinear estimation of the activation energy (Ea), the frequency factor (A) and the initial drug concentration (C_0) expressed as the percentage of drug remaining. Also, the R® program was used for deriving 95% confidence interval bootstraps, i.e. basic, percentile, and bias-corrected and accelerated (BCa), for the parameter estimates: C_0, Ea, A and shelf-life, ($t_{90\%}$), i.e. the time period required for a drug to degrade to 90% remaining at 25 °C.

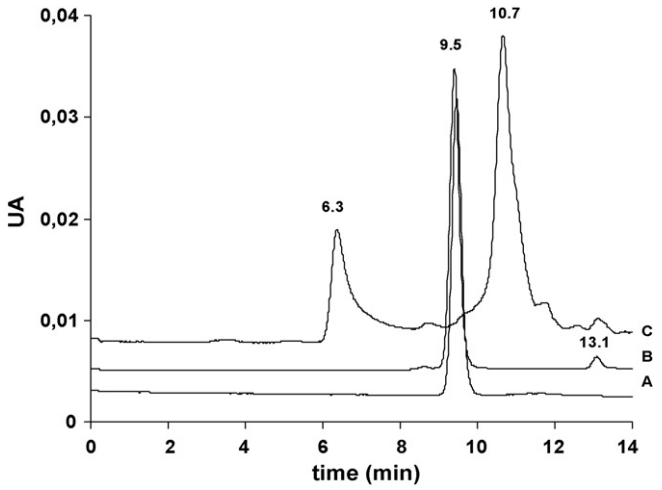

Fig. 1. Chromatograms of a human insulin pharmaceutical preparation: (A) standard sample; (B) sample stored under isothermal (60 °C, 10 days) and non-isothermal (60 °C, 5 days) conditions; (C) sample stored under non-isothermal conditions (81 °C, 8.5 days). Peak identification: 6.30 min, covalent insulin dimer; 9.5 min, human insulin; 10.7 min, product from proteolysis; 13.1 min, desamido-B3 insulin.

3. Results and discussion

3.1. Isothermal studies

An apparent first-order degradation process was observed at all temperatures studied (20–60 °C). To verify the validity of the kinetic model and measure linearity, correlation coefficients (r) and standard errors were calculated. The rate constants were obtained from the slopes of the semi-log plots of concentration versus time by linear regression analysis, the correlation coefficient being greater than 0.95. The residual plots showed the absence of trends or correlations, the signs test [19] confirmed the validity of proposed model and therefore, the residuals represent only the experimental error.

A linear Arrhenius plot ($r = 0.9998$) suggested the same insulin degradation mechanism within the studied temperature range (Fig. 1), the desamido B3 insulin being the main degradation product, although the covalent insulin dimer (CID) was also detected (<0.5%). The insulin degradation products and mechanism are perfectly known and published for many authors, for example, Brange [20] have published various paper related with the chemical stability of insulin, including isolation, characterization and identification of insulin transformation products during storage of pharmaceutical preparations. In our case, the desamido B3 insulin and CID have been previously identified [21].

The Ea and A obtained from Arrhenius plot were equal to 25.45 Kcal/mol and $1.01 \times 10^{14}\,h^{-1}$, respectively, similar to that reported for degradation of different insulin preparations [20,22–24]. The uncertainties given as 95% confidence intervals were calculated from the residual standard deviation by the standard expression [19] are shown in Table 1. The uncertainties are quite large due to the small number of degrees of freedom ($\upsilon = 2$) based only on the scatter in the Arrhenius plot.

The estimated shelf-life ($t_{90\%}$) at 25 °C was 199.1 days, assuming that the activation energy remains constant over the temperature range 25–60 °C. In order to estimate the uncertainty in the calculation of $t_{90\%}$, the 95% confidence intervals were calculated to Ln K, obtaining the values of 145.3 and 272.6 days, respectively. These confidence intervals are very large since there is a coupling of the uncertainty in the prediction due to fitting the rate constants at each temperature and the temperature dependence for extrapolation to storage temperature.

3.2. Non-isothermal studies

The principal challenges of non-isothermal studies are generating the programmed temperature rise and the subsequent data analysis. In the first case, the difference between the theoretical (0.250 °C/h) and experimental (0.2505 °C/h) linear heating-rate constants was less than 0.5%, therefore the temperature control was excellent throughout the experiment.

Like the traditional isothermal stability study, the non-isothermal experiment is also based on the Arrhenius equation, but the accuracy and precision of its estimates depend largely on the experimental conditions. So, 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 [1,5,11,14]. The first step is to determine the suitable temperature range where the Arrhenius relationship would be valid. Initially, the temperature range was set up to be from 30 to 90 °C and a linear heating rate of 0.25 °C per hour to facilitate greater degradation, the experimental period being 10 days. In these conditions, temperatures higher than 70 °C were ruled out because the degradation process did not remain constant, due mainly to insulin aggregation, where the covalent dimer formation becomes increasingly favoured relative to deamidation. This fact was confirmed by HPLC and MALDI-TOF mass spectrometry results (Figs. 1 and 2). The high temperature can lead to the formation of soluble or insoluble aggregates, covalent or non-covalent, which can vary in size from dimer to large fibrils [25]. Aggregation can occur through multiple mechanisms that are sometimes difficult to elucidate. In our case, the covalent insulin dimer (CID) was detected from 70 °C upwards, although its formation seems to follow a nucleation process. Oliva et al. [21] reported that the combination of shaking and high temperature (60 °C) promotes insulin dimer formation fitting well with the Prout-Thompkins nucleation model. At the same time, a second degradation pathway was observed, involving cleavage of the peptide bond, i.e., proteolysis in different positions. In this case, various peaks with masses around 2400–3400 Da were detected by MALDI-TOF mass spectrometry (Fig. 2). To confirm this data, the molecular weight values of the different fragments were used to identify the possible sequence (i.e. primary structure) using Web-bases search engines such as SWISS-PROT or TrEMBL database. In our study, the fragment corresponding to both insulin chain were identified (this fact implies the cleavage into A- and B-chains as result of disulfide lysis). Degradation of protein disulfide bonds in a variety of products is a well-established phenomenon [26], but the mechanism of the S–S bond lysis is under dispute [27,28]. Also, other fragments were identified as result of the autoproteolytic cleavage of both chains and ones were not identified.

However, it appears that the predominant pathway shows strong temperature dependence. Thus, proteolysis occurs more rapidly at temperatures higher than 85 °C where the dimer could also undergo a proteolysis reaction. MALDI-TOF mass spectrometry analysis of a sample stored at 88 °C showed peaks at masses between 6000 and 8000 Da. In accordance with all these data, a second experiment was conducted in the temperature range from 30 to 70 °C, while the rest of the conditions were similar to those described above.

For data analysis, the integral approach proposed by Yoshioka et al. [5], was used to calculate the Arrhenius parameters, which involves a direct nonlinear estimation of the Arrhenius parameters from Eq. (1) without preliminary mathematical treatment; providing reliable estimates with smaller deviations and biases. The numerical integration method is free from bias because it fits the values of Ea and A directly to the data in a single step, rather than fitting the data to some function and then obtaining Ea and A separately. It also takes into account the scatter in the original data,

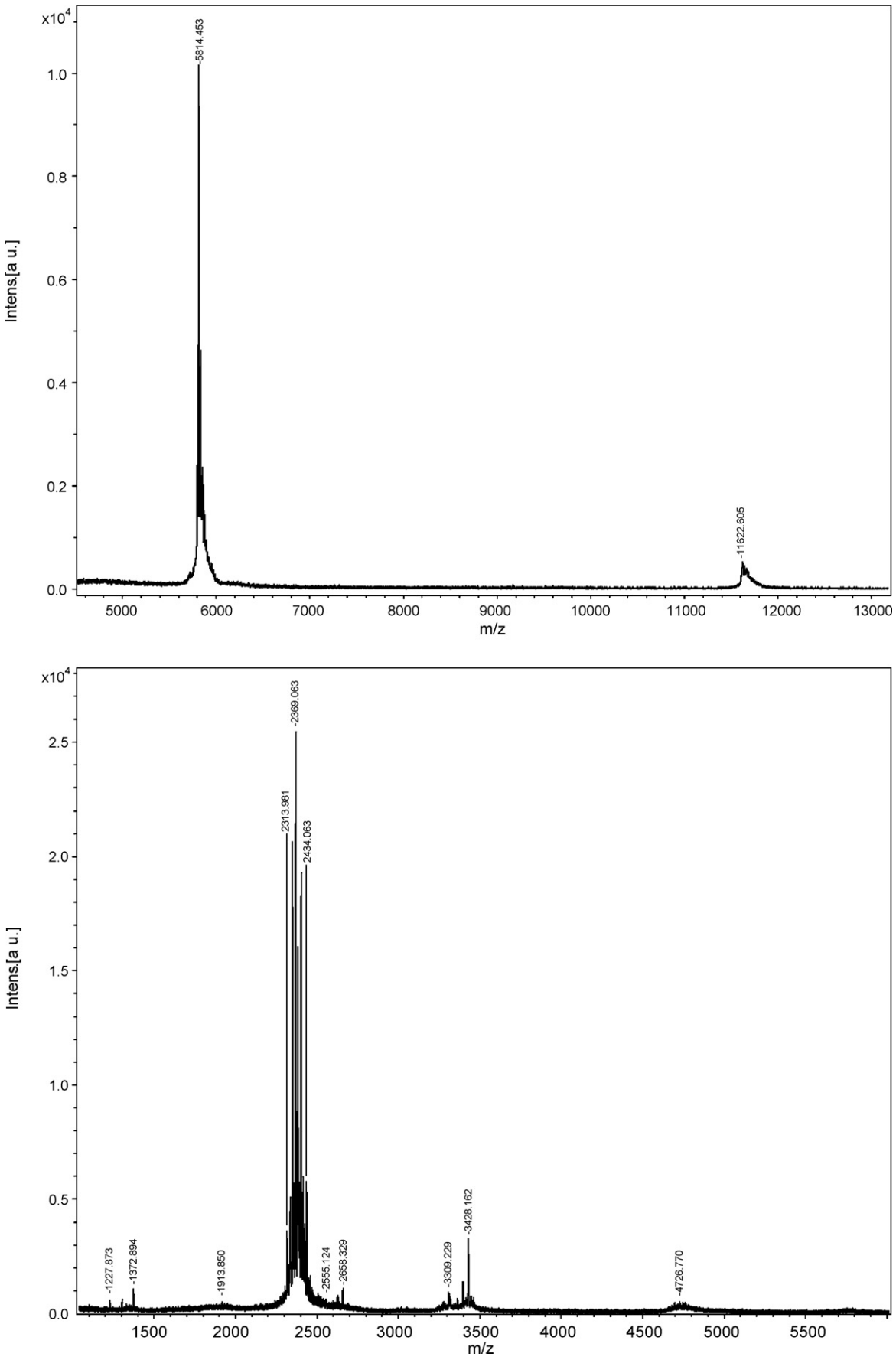

Fig. 2. MALDI-TOF mass spectra of the human insulin. (Upper) Sample stored at 70 °C showed the presence of monomer (m/z = 5814.4) and covalent insulin dimer (m/z = 11622.6). (Lower) Sample stored at 85 °C revealed various peaks from proteolysis ($m/z \approx 2400$).

Table 1
Comparison of the kinetic parameters (Ea, A, Co) and shelf-life ($t_{90\%}$) obtained by the isothermal and non-isothermal method.

Non-isothermal approach						Isothermal approach	
Parameter	Estimates	CI asymptotic[a]	Basic[b]	Percentile[b]	BCa[b]	Estimates	95% CI[c]
Co (%)	99.40	99.11–99.69	99.12–99.67	99.14–99.68	99.14–99.69	–	–
Ln A (h^{-1})	27.94	24.15–31.73	24.28–31.43	24.44–31.59	24.42–31.56	32.24	31.49–32.98
Ea (cal/mol)	22,879	20,371–25,388	20,464–25,191	20,567–25,294	20,557–25,289	25,455	24,987–25,922
$t_{90\%}$ (25 °C, days)	191.4	125.5–290.9	122.4–286.9	127.6–299.1	127.0–297.0	199.1	145.3–272.6

[a] 95% confidence interval based on asymptotic standard error.
[b] Different 95% confidence interval Bootstrap.
[c] 95% confidence intervals based on typical standard deviation.

which is inherently impossible using the two-step isothermal and derivative approaches. In our cases, the R® statistical program (version 2.7.1) was used to fit the degradation profile of insulin. Both Ea and *A* were determined by an iterative nonlinear least-square method (Fig. 3a). The output from the R® program generated an optimal value both for the activation energy of 22.88 Kcal/mol and $1.36 \times 10^{12}\ h^{-1}$ for the frequency factor. These values are in good agreement with similar data found in the literature [20,22–23]. Therefore, this method also gives a reliable estimate of all parameters, and the most realistic estimate of the uncertainties involved (Table 1) due to the higher number of degrees of freedom. These results are reflected in the goodness of fit between experimental values and the kinetic model chosen. Also, the assumption of normally distributed errors was checked by examining the residuals and Q–Q (quantile–quantile) probability plots (Fig. 3b). The plot of residuals shows that the normality and independence of the errors is preserved.

For nonlinear models, estimation of confidence intervals is not straightforward, and they are not necessary symmetrical; the extent of asymmetry depends on the nonlinearity of the function and the quantity of data [29]. The first and easiest method to determine the confidence intervals is to use asymptotic standard errors, but this may significantly underestimate the confidence intervals. A more reliable method would be to search for values of each parameter causing the objective function to be greater than its minimum by the amount of some critical value given by χ^2or *F*-distribution. This is also very tedious. By far the best method to evaluate confidence intervals applies the Monte Carlo technique and is called the "bootstrap". Today the lowest-cost computers and software have free statistical packages such as Solver® in Excel® or R® program, which have gained acceptance and popularity in the field of applied statistics.

In this case, the software R® program was used for deriving 95% confidence interval bootstraps for the following parameter estimates: C_0, Ea and *A*. In total, 9999 bootstraps were performed on these parameters, and the different bootstrap confidence intervals are shown in Table 1. The bootstrap replicates are displayed in Fig. 4. The symmetry of the parameter estimates indicates a normal distribution. The length and shape of the different confidence intervals show no differences between them, since all approaches give closely similar uncertainty estimates and the results from the different runs lie within each another's uncertainty limits. These confidence intervals are very close to those obtained using the asymptotic standard error method and, therefore, the assumption of normally distributed errors and the kinetic model chosen are correct.

The $t_{90\%}$ was calculated by the standard procedure assuming first-order kinetics at 298.15 K, obtaining a value of 191.4 days.

The bootstrap technique was again used for deriving confidence intervals for $t_{90\%}$, and confirms that its distribution is also normal. The bootstrap replicates for $t_{90\%}$ are shown in Fig. 5. Note the asymmetry of the parameter estimates; the curves in Fig. 5a would appear symmetrical since a normal distribution was assumed. In

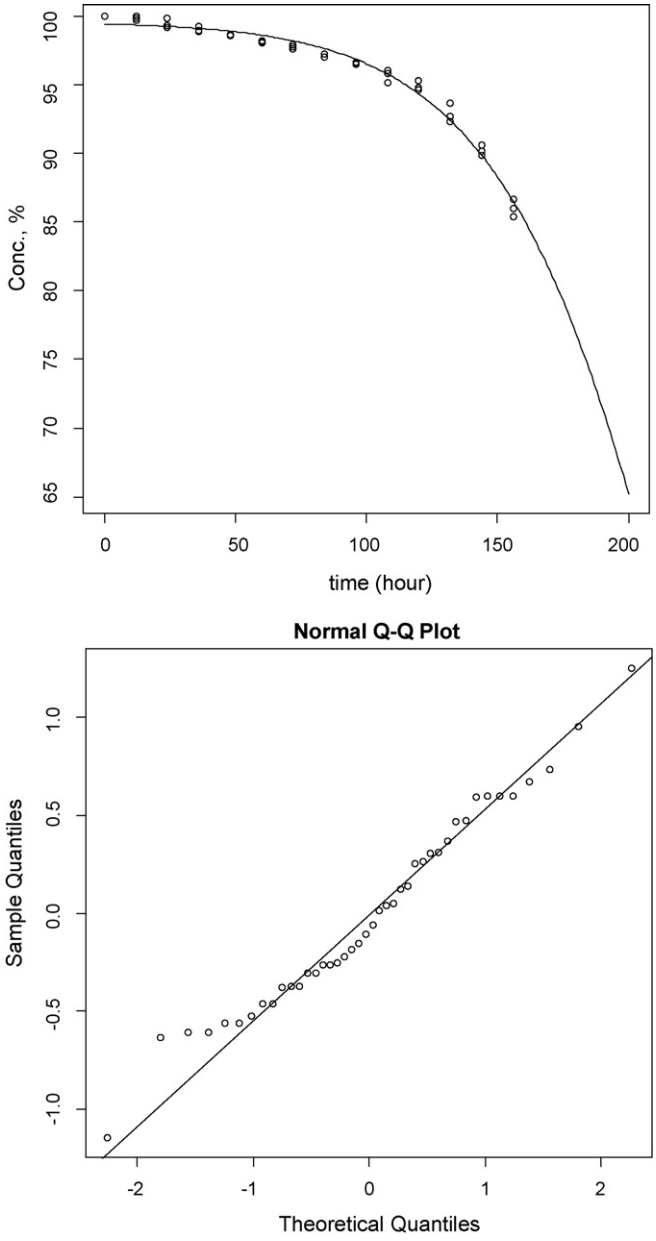

Fig. 3. (Upper) Variation of the insulin concentration expressed as percentage remaining (o) versus time, considering an experimental linear heating-rate of 0.2505 °C/h. Data were fitted using the R® Program. (Lower) QQ-plot (i.e. quantile–quantile) of the residuals showing a normal distribution line.

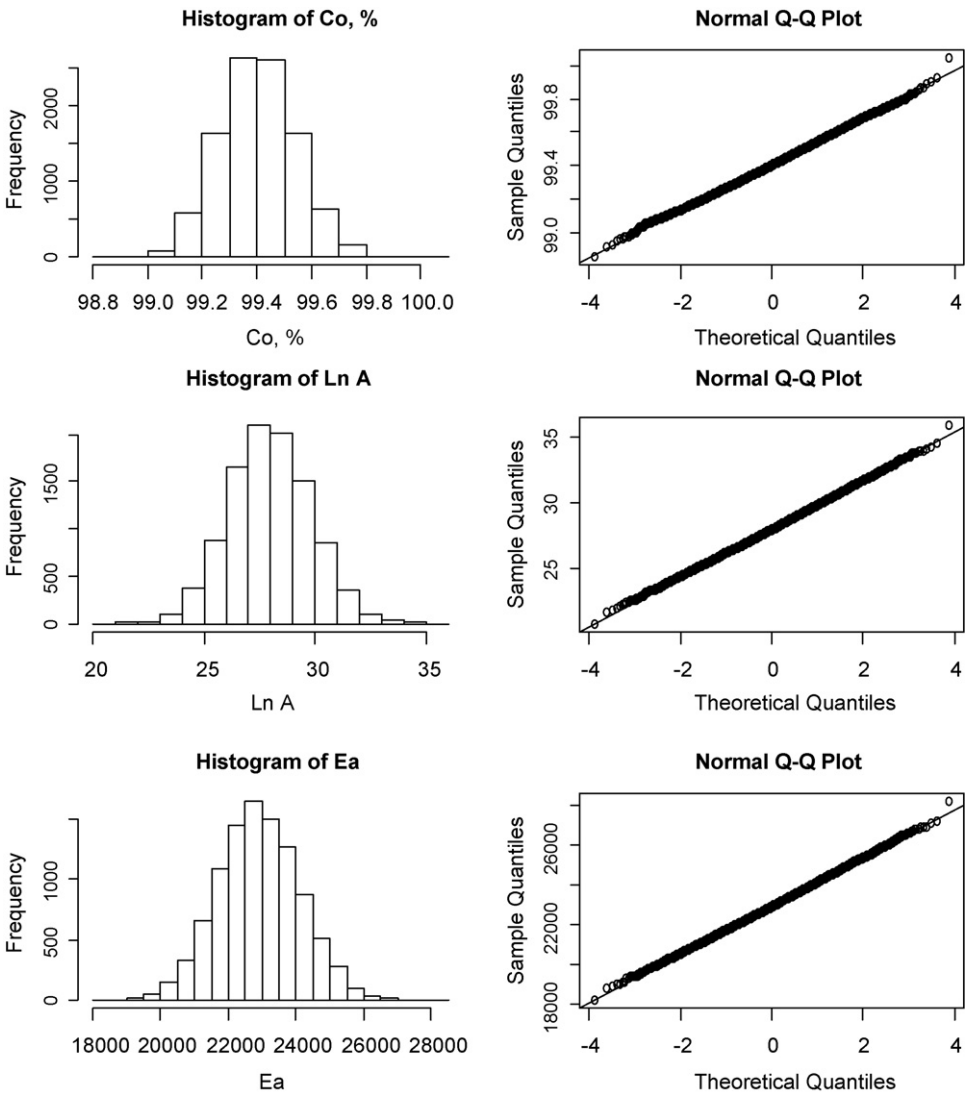

Fig. 4. Histograms of 9999 bootstrap iterations and the quantiles of standard normal plots for the Co (upper), Ln A (medium) and Ea (lower) parameters estimated by the integral approach.

fact, this is not true, and the distribution is significantly skewed to the right side. This could suggest that the $t_{90\%}$ parameters follow a log-normal distribution (Fig. 5b). In this case, the asymptotic and bootstrap confidence intervals were very similar.

3.3. Comparison of isothermal and non-isothermal method

The results shown in Table 1 indicate that the estimated kinetic parameters in non-isothermal conditions were comparable to those obtained in isothermal conditions. The percentage difference in activation energy was observed to be less than 10.1%, whereas the difference was 98.6% 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.

The estimated shelf-life in non-isothermal conditions was quite close to the value derived from isothermal data, a difference of 7.7 days, although the 95% confidence intervals were slightly higher (Table 1). This variation is due to the differences in the estimation method and the nature of the experimental errors.

The results obtained point to a situation in which the Ea and A parameters for two given experiments could differ greatly, 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. An explanation consistent with this could be the high correlation between the estimates of Ea and A, attributed to the nature of the Arrhenius equation. This fact can be verified if the asymptotic correlation matrix is analysed. Thus, small variations in one parameter will considerably affect the other; therefore the $t_{90\%}$ will change. In general, this can be considered a specific problem of non-isothermal studies, but not as a problem of the data-fitting method.

In any case, the shelf-life estimated at 25 °C was higher that manufacturer's recommendation (28 days). However, the vast majority of biopharmaceuticals require a low temperature environment, typically 2–8 °C, and a cold storage chain thus needs to be maintained.

The advantages of non-isothermal methods over the traditional isothermal method, such as the minimum amount of material needed, small number of experiments and experimental running time are evident, and obtaining similar results with good accuracy and precision. For example, the experimental period needed by this non-isothermal method was about four times shorter than that needed in more rapid experiments under isothermal conditions.

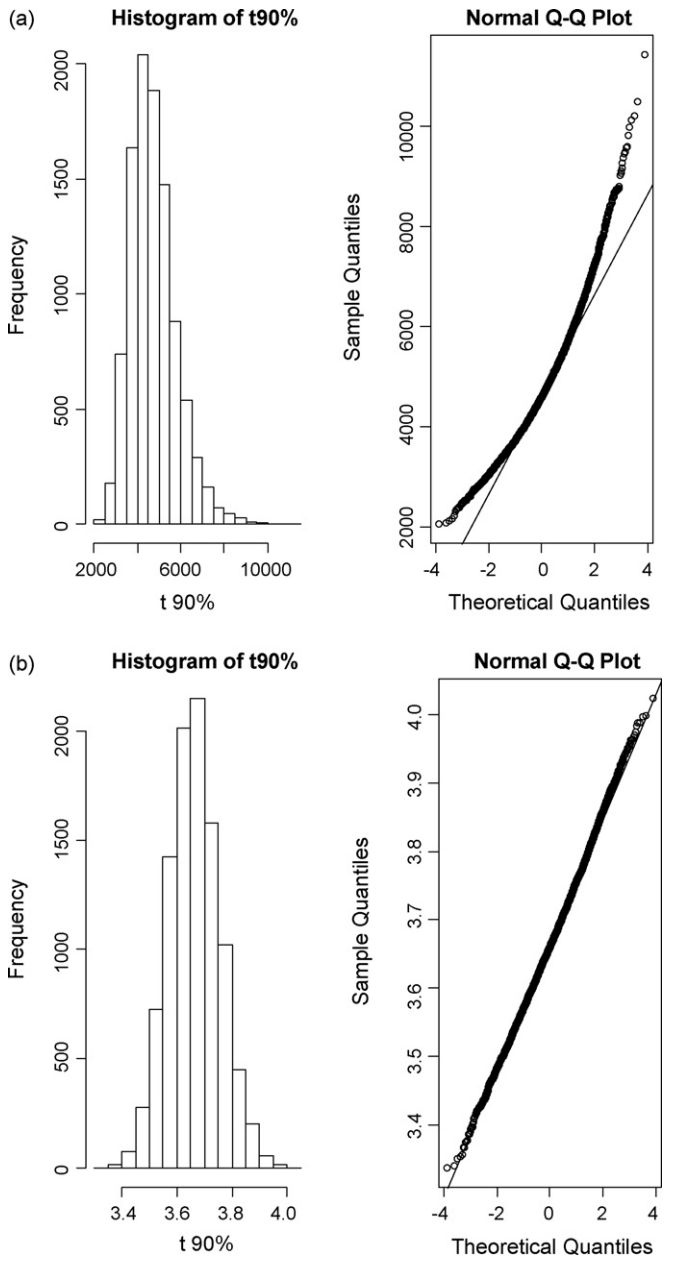

Fig. 5. Histograms of 9999 bootstrap iterations and the quantiles of standard normal plots for the shelf-life, assuming a normal (left) and log-normal distribution (right).

4. Conclusions

The linear Arrhenius plot seems to indicate that the degradation mechanism and kinetics do not change with temperature in the range 20–70 °C, the desamido B3 insulin, CID and derivatives from proteolysis being the main degradation products detected. At first, the similarity of Ea values obtained under each set of conditions with a difference lower than 10.1% seems to confirm this despite a marked difference in the frequency factors.

The non-isothermal method seems to be superior to the isothermal method in the sense that both Ea and *A* can be estimated by a single experiment, providing information on the dependence of degradation rate on temperature and allows approximate prediction of drug stability. The bootstrap technique can be used for estimating confidence intervals of Arrhenius parameters and shelf-life although the underlying distribution function of the parameters is unknown. Also it is evident that this technique can supplement and extend conventional statistical methods.

In summary, these results indicate that the non-isothermal methodology used in this study may be used to extrapolate peptide and protein stability and, therefore, to estimate the shelf-life for pharmaceutical preparations of other peptides and proteins, saving time, samples and experimental effort.

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