Talanta 94 (2012) 158–166

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

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

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ARTICLE INFO

Article history:
Received 27 October 2011
Received in revised form 1 March 2012
Accepted 5 March 2012
Available online 10 March 2012

Keywords:
Peptides
Stability
Arrhenius
Bootstrap
Regression method

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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doi:10.1016/j.talanta.2012.03.011

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 $n-1$

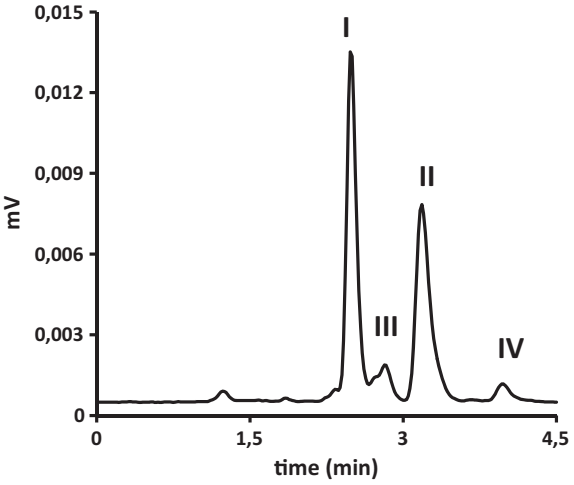

Fig. 1. Chromatogram of a CCK-4 sample stored at 60 °C for 5 days, showing the three degradation products, identified as cyclic dimer (II), whereas peaks III and IV derived from the cyclic dimer after the loss of one (III) or two Phe-NH_2 (IV) residues.