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Pre-study and in-study validation of a size-exclusion chromatography method with different detection modes for the analysis of monoclonal antibody aggregates

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ABSTRACT

Size exclusion chromatography (SEC) with different detection modes was assessed as a means to characterize the type of bevacizumab aggregate that forms under thermal stress, quantitatively monitoring the aggregation kinetics.

The combination of SEC with light-scattering (SEC/LS) detection was validated using in-study validation process. This was performed by applying a strategy based on a control chart to monitor the process parameters and by inserting quality control samples in routine runs. The SEC coupled with a differential refractive-index detector (SEC/RI) was validated using a pre-study validation process in accordance with the ICH-Q2 (R1) guidelines and in-study monitoring in accordance with the Analytical Target Profile (ATP) criteria. The total error and β-expectation tolerance interval rules were used to assess method suitability and control the risk of incorrectly accepting unsuitable analytical methods.

The aggregation kinetics data were interpreted using a modified Lumry-Eyring model. The true order of the reaction was determined using the initial-rate approach. All the kinetic data show a linear Arrhenius dependence within the studied temperature range. The Arrhenius approach over-predicted the aggregation rate for 5 °C, but provides an idea of the aggregation process and amount of aggregate formed. In any case, real-time stability data are necessary to establish the product shelf-life.

1. Introduction

Antibody therapeutics is undergoing rapid growth rates, the major areas of application being cancer and various immunological disorders. Bevacizumab (Avastin^{R}, Genetech, San Francisco, USA) is a recombinant humanized monoclonal IgG1 antibody that prevents or reduces the formation of blood vessels (angiogenesis), thereby preventing or reducing metastatic disease progression [1]. It has been also shown to be effective as an adjunct treatment for neovascularization of the iris and neovascular glaucoma with or without vitreous hemorrhage [2]. The successful application of this important class of drugs requires avoiding any form of degradation.

For this, the main product characteristics to be monitored are aggregate and fragment content, glycosylation pattern and charged isoforms. To define the identity or purity of biopharmaceutical drugs requires methodologies that differ from classical pharmaceutical practices [3]. However, due to the complexity of protein drugs, different analytical methods must be applied in protein characterization and development of stability-indicating assays. The effect of heat, shear, surface phenomena, and solvent additions on the native-state protein should also be studied [4]. All the techniques are expected to detect, quantify and distinguish different forms of the active ingredients and their degradation products.

The standard method used in biopharmaceutical quality control (QC) for monoclonal antibody (mAb) aggregate and fragment analysis is size exclusion chromatography (SEC). Its main advantage is the mild elution conditions that allow protein characterization with minimal impact on conformational structure and local environment [5]. The potential and applications of this analytic technique in the characterization of biopharmaceutical drugs was recently reported by Fakete et al. [5]. SEC in combination with light-scattering (SEC/LS) detection offers an easy, accurate and reliable alternative technique to assess the association of macromolecules in solution [6,7]. In some instances it can provide information regarding the conformation of a protein (folded or unfolded), if the

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molecular mass derived from light-scattering and elution position are compared [8].

The validation of quantitative analytical methods is vital to check the safety and efficacy of biopharmaceutical drugs. Many regulatory organizations have addressed this issue in the chemical and pharmaceutical industry (e.g. International Conference on Harmonization-ICH, the Food and Drug Administration-FDA and Eurachem). Analytic validity is assessed at two levels. The "pre-study" validation aims to show that the method is able to achieve its objectives, whereas "in-study" validation verifies that the method remains valid over time by including quality control samples in routine runs [9].

The first step is to establish the Analytical Target Profile (ATP), which defines what the method is required to measure (acceptance criteria) and to what exactness (performance level characteristics—such as precision, accuracy, working range, sensitivity—and the associated performance criteria) [10]. However, a control strategy for the method has to be implemented, to assure that the method will perform as intended on a routine basis. For this, the use of control charts such as X-bar or moving range (MR) are a good option [11].

The aim of our research was to check the suitability of the SEC, combined with different detection modes in the characterization of the mAb aggregation process under thermal stress. The combination of SEC with light-scattering (SEC/LS) detection was used in the characterization process (i.e., identity of starting proteins, products and intermediates of protein aggregation), whereas the SEC coupled with a differential refractive-index detector (SEC/RI) was used to obtain kinetic data. For this, (i) the first method was validated using an in-study validation process. (ii) the second was validated using a pre-study validation process in accordance with the ICH-Q2 (R1) guidelines [12] and in-study validation procedure, applying ATP criteria. The total error and β-expectation tolerance interval rules were used to assess method suitability and control the risk of incorrectly accepting unsuitable analytical methods. (iii) Kinetic modeling was applied to predict the stability of mAb under thermal stress and its shelf-life. All these aspects were analyzed using a mAb, bevacizumab, as model molecule.

2. Materials and methods

2.1. Materials

The proteins alcohol dehydrogenase and carbonic anhydrase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bevacizumab (Avastin® , Genentech Inc.) was supplied as a 25 mg/mL solution in phosphate buffer (pH = 6.2) containing 159 mM α,α-trehalose dehydrate and 0.04% w/v polysorbate 20. Deionized water was purified in a MilliQ plus system from Millipore (Molsheim, France), prior to use. All other chemicals and reagents were HPLC grade. All solvents were filtered with 0.45 μm (pore size) filters (Millipore) and degassed.

2.2. SEC/RI system

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 differential refractive index (RI) detector (Waters model 2414). Elution was performed at room temperature in a Protein KW-804 column (8 × 300 mm, Waters). The data was collected and analyzed using the Millennium32® chromatography program (Waters). The mobile phase was phosphate-buffered saline (300 mM NaCl, 25 mM phosphate, pH 7.0) at a flow rate of 1.0 mL/min, and injection volume 25 μL.

2.3. SEC/LS system

The multi-angle laser light-scattering detector (miniDawn, Wyatt Tech.) was placed downstream of the column and upstream of the RI detector. To reduce baseline noise a pulse dampener (Alltech Associates, USA) was connected downstream of the pump and two 25 mm high-pressure filters (Millipore) with respectively 0.22 and 0.1 μm pores were used for on-line filtration of the mobile phase.

The column and other chromatographic conditions were identical to those used for the SEC system mentioned above. A 100 μL sample of each solution was injected into the system, and data collection and analysis were performed using Wyatt Technology's Astra ™ program. A differential refractive index increment (dn/dc) of 0.184 ± 0.003 mL/g was estimated [13]. The weight-average Mw was calculated through the Rayleigh-Gans-Debye approximation [14].

2.4. Stability

Samples of bevacizumab were stored in an oven (BR-UT 6000 Model, Heroes Instruments, Germany) at different temperatures (25, 40 and 50 °C) and in a refrigerator at 5 °C (2–8 °C) for 18 months. Aliquots were removed at appropriate intervals, and analyzed the same day in duplicate. In all cases, the percentage of each species was calculated according to the ratio of the area under the corresponding peak to the total area.

3. Results and discussion

3.1. In-study validation of SEC/LS method

In a previous work [13], the SEC/LS method was validated using the in-study validation procedure. The X-bar and MR-control charts showed that the analytic method was in-control (i.e., within acceptable bounds) and stable, although greater control of precision was required.

In the course of this study, experiments were performed to analyze the effect of the initial concentration as a function of temperature, called the initial-rate method for reaction-order determination. During this time, 8 control samples were analyzed, implying that the standard deviation and mean of the process has to be re-calculated (Fig. 1), called second phase (n = 38). During this phase, a new column and a different batch were used. The individual measurements show an apparently lower dispersion with respect to the first phase, but the process mean was maintained (i.e., accuracy) since the analysis conditions were the same. However, precision improved to a value of 1.38 kDa versus 1.55 kDa for the first study phase. The sudden change observed from phase #2 in the MR- control chart may be related to the column change (new column and different batch), since the column performance and efficiency werehigher (Fig. 1), the overall uncertainty calculated using the Bayesian posterior distribution [15] was lower than 2% (see Fig. 2).

To determine whether the process meet the required capability requirement and ran under the desired quality conditions, the index C_{pm} was computed as 1.83 [1.40–2.26]. However, this result does not imply a change in capability, since the lower confidence bound C on C_{pm} was 1.26, a similar value to those obtained in the first phase. Therefore, the process is still considered "capable" ($1 \leq C_{pm} < 1.33$) [16].

Two proteins with different Mw were also used as internal controls: Carbonic anhydrase with a nominal Mw of 29.1 kDa was analyzed monthly, whereas the alcohol dehydrogenase with a nominal Mw of 141 kDa, similar to bevacizumab, was analyzed ran-