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Capillary isoelectric focusing method development and validation for investigation of recombinant therapeutic monoclonal antibody

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ABSTRACT

Capillary isoelectric focusing (cIEF) is a basic and highly accurate routine analytical tool to prove identity of protein drugs in quality control (QC) and release tests in biopharmaceutical industries. However there are some "*out-of-the-box*" applications commercially available which provide easy and rapid isoelectric focusing solutions for investigating monoclonal antibody drug proteins. However use of these kits in routine testings requires high costs.

A capillary isoelectric focusing method was developed and validated for identification testing of monoclonal antibody drug products with isoelectric point between 7.0 and 9.0. A method was developed providing good pH gradient for internal calibration ($R^2 > 0.99$) and good resolution between all of the isoform peaks ($R = 2$), minimizing the time and complexity of sample preparation (no urea or salt used). The method is highly reproducible and it is suitable for validation and method transfer to any QC laboratories. Another advantage of the method is that it operates with commercially available chemicals which can be purchased from any suppliers. The interaction with capillary walls (avoid precipitation and adsorption as far as possible) was minimized and synthetic isoelectric small molecular markers were used instead of peptide or protein based markers.

The developed method was validated according to the recent ICH guideline (Q2(R1)). Relative standard deviation results were below 0.2% for isoelectric points and below 4% according to the normalized migration times. The method is robust to buffer components with different lot numbers and neutral capillaries with different type of inner coatings. The fluoro-carbon coated column was chosen because of costs-effectivity aspects.

1. Introduction

The emerging market of biosimilar monoclonal antibodies (MABs) generated need for more and more accurate determination of similarity of biosimilar candidates to the originator's products [1].

Characterization of a monoclonal antibody is a very difficult task in every analytical aspect, due to the various post-translational modifications such as glycosylation, phosphorylation or lysine clipping. During the whole process and storage the protein drug molecules may suffer from many physico-chemical stresses, resulting in the formation of charge variants like oxidation of tryptophan or deamidation of asparagine [2]. All of these modifications (both biological and chemical) contribute to the full charge profile of the protein. Some of these charge variants may have possible effect on biological activity of the molecule. Glycosylation profile of monoclonal antibody highly influences the biological activity of the protein [3,4]. For example, there are several evidences of the negative effect of the acidic charge variants on the antigen-binding capability of monoclonal antibody [5].

Monitoring charge variants and product related impurities during storage is essential from the point of view of safety and activity [6], consequently it is one of the most important analytical aspect of biosimilar development.

The most advanced techniques such as liquid chromatography coupled to tandem mass spectrometry [7,8] are powerful tools for characterization of biological drug products, but are difficult to implement into the routine analysis and regulated quality control.

Capillary isoelectric focusing (cIEF) is a powerful tool to characterize charge profile of the monoclonal antibodies, because different charge variants can be distinguished according to their isoelectric points using internal calibration [9]. As cIEF provides

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very high reproducibility and specificity, this is one of the most popular tools for monoclonal antibody analysis.

There are some different approaches of isoelectric focusing in capillaries [10–12,19]. The basis of the methods is the same: the separation according to the p*I* takes place in pH gradient formed by carrier ampholytes in silica capillary (coated or uncoated). The separations can be performed in two steps consecutive of the focusing and the separation or in one step only [12].

According to the conventional approach the peaks can be detected in "continuous" mode through a capillary window by UV detector, or by on-capillary-detected solutions called imaged [13,14] capillary isoelectric focusing. These later techniques operate just with the focusing step and no mobilization is required providing fast separations. Other solution needs special instrumentation [15,16], but cannot be implemented into routine analysis.

In this study we intended to adjust a high resolution and highly reproducible capillary isoelectric focusing application for identification testing of monoclonal antibody with isoelectric points between 7.4 and 8.0. Moreover our aim was to make this method capable of analysing different MABs within p*I* range 7–9.

2. Materials and methods

2.1. Materials

Hydroxypropyl methylcellulose (HPMC), iminodiacetic acid (IDA), arginine (ARG), Pharmalytes (pH 5–8 and pH 3–10), phosphoric acid, sodium hydroxide, acetic acid, Tris buffer, carboxypeptidase-B (CPB) enzyme and UV–visible synthetic cIEF markers were purchased from Sigma–Aldrich (St Louis, MO, USA). Commercially available monoclonal antibodies were purchased from Hoffmann La Roche (Basel, Switzerland) and Janssen Biotechnology Inc. (Horsham, PA, USA).

2.2. Instrumentation

All measurements were performed on Beckman Coulter (Hercules, CA, USA) PA800 and PA800 Plus capillary electrophoresis systems. 50 μm inner-diameter fluorocarbon coated capillaries (μSil-FC) were purchased from Agilent Technologies (Santa Clara, CA, USA) and 50 μm inner-diameter polyacrylamide (PAAM) coated capillaries were purchased from Beckman Coulter and Sepax Technologies (Newark, DE, USA). The polyimide coating was burnt on both ends of the capillaries by Microsolv CE Window Maker apparatus (Microsolv, USA Monmouth).

2.3. Buffers and solutions

40 mM phosphoric acid solution was used as anode buffer, 80 mM sodium hydroxide solution was used as cathode buffer and 100 mM acetic acid was used as mobilizer and rinse solution. 1% (m/v) HPMC solution was prepared as separation gel buffer, 500 mM arginine solution as anodic stabilizer and 20 mM iminodiacetic acid as cathodic stabilizer according to Mack et al. [17]. The formulated MAB solution was diluted with capillary electrophoresis (CE) grade purified water to 0.5 mg/ml concentration to minimize any interference with salts.

2.4. Sample solution

The investigated sample solution contained 46% (v/v) HPMC solution, 41% (v/v) 0.5 mg/ml MAB solution, 3.2% (v/v) mixture of Pharmalyte 3–10 and 5–8 (1:5), 5.5% (v/v) arginine solution, 0.9% (v/v) iminodiacetic acid solution and 0.9% (v/v) of each p*I* markers. In the case of blank and system suitability solution 41% (v/v) purified water was used instead of the 0.5 mg/ml MAB solution. The same sample solution setup was applied for all of the samples used for specificity measurements.

2.5. General setup

The basic setup, which was not modified during method development, was the following: the amount of the cathodic and anodic stabilizers (arginine and iminodiacetic acid), the percentage of HPMC in the solution and the amount of p*I* markers in each case were the same described in Section 2. The injection was performed during 60 s with 20 psi hydrodinamical pressure, the focusing step for 10 min and the mobilization step for 20 min were used. The chemical mobilization was done by changing catholyte buffer to 100 mM acetic acid. 20 °C capillary temperature was used according to Cao et al. [9] and the samples were stored at 10 °C.

2.6. Marker selection

Accurate p*I* determination is based on the p*I* markers with well-defined isoelectric points. However, most of the recent cIEF methods for therapeutic proteins used peptide-based isoelectric markers [9,17]. Synthetic p*I* markers were chosen in our experiments, due to their wide p*I* range, good UV absorbance, easy accessibility from the market and minimal interaction with the capillary walls. During development three synthetic markers were chosen with different p*I* below and over the p*I* of the analyte indicating the goodness of the pH gradient by the R^2 coefficient of the *migration time–pI* function. As the analyte charge distribution is between 7.4 and 8.0 markers were selected from *Sigma* with p*I* 7.0, 8.4 and 9.0.

2.7. Anolyte, catholyte and chemical mobilization buffer

40 mM phosphoric acid was used as anolyte, 80 mM sodiumhydroxide as catholyte and 100 mM acetic acid as chemical mobilizer. No difference was detected between the different concentrations (data not shown). Fresh buffers were used after every third injections to avoid contamination and dilution of electrolytes.

3. Results and discussion

3.1. Method development

Our aim was to achieve the maximum resolution combined with as short migration time as possible. The setups with shorter migration times were chosen. In this study a commercially available human IgG1 type monoclonal antibody with five main isoforms was chosen as a model protein for the investigations.

As indicator of the optimal Pharmalyte ratio the goodness of migration time–isoelectric point plot was chosen. The regression coefficient of the plot (R^2) was investigated and 0.99 as minimum value was determined (data not shown). Runs with R^2 value lower than minimum were rejected.

According to Refs. [9,17,18] the following critical parameters were chosen for method development: focusing and mobilization voltage, amount of salt and urea in the sample. In every case 10 min focusing voltage was used according to Cao et al. [9] to achieve short separation.

Equal resolutions between all the peaks were aimed during development. Resolutions between peaks were determined by Eq. (1):

$$R = \frac{t_{m(N+1)} - t_{m(N)}}{pI_{(N)} - pI_{(N+1)}}$$