Development of a Cation-Exchange pH Gradient Separation Platform

Shanhua Lin,[1] Julia Baek,[1] Wim Decrop,[2] Srinivasa Rao,[1] Yury Agroskin,[1] and Chris Pohl[1]
[1]Thermo Fisher Scientific, Sunnyvale, CA; [2]Thermo Fisher Scientific, Amsterdam, Netherlands

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Overview

Purpose: Generate a linear pH gradient for MAb charge variant separation.

Methods: Eluent A and eluent B each contains multi-component zwitterionic buffer species. Linear gradient was run from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). Online monitoring of the mobile phase pH value confirmed that a linear pH gradient was achieved.

Results: This linear pH gradient enables the high resolution separation of MAb charge variants.

Introduction

MAbs can be highly heterogeneous due to modifications such as sialylation, deamidation and C-terminal lysine truncation. Salt gradient cation exchange chromatography has been used with some success in characterizing MAb charge variants. However, additional effort is often required to tailor the salt gradient method for an individual MAb. In the fast-paced drug development environment, a more generic platform method is desired to accommodate the majority of the MAb analyses.

In 2009, Farnan and Moreno reported a method to separate MAb charge variants using pH gradient ion-exchange chromatography. The buffer employed to generate the pH gradient consisted of piperazine, imidazole, and tris, covering a pH range of 6 to 9.5. While good separation was observed, the slope of the pH increase was shallow at the beginning and steep towards the end (Ref. 1). In this study, we present a novel pH gradient method for cation exchange chromatography. This method features a multi-component buffer system in which the linear gradient was run from 100% eluent A (low pH buffer) to 100% eluent B (high pH buffer). Using an online pH meter, it was confirmed that a linear pH gradient was achieved. Furthermore, a plot of measured pH values of model proteins versus their pI values exhibited a high correlation. Once the approximate pH elution range of the target MAb has been established in the initial run, further optimization of separation can simply be achieved by running a shallower pH gradient in a narrower pH range.

Methods

Sample Preparation

All standard proteins were purchased from Sigma-Aldrich©. Harvest cell culture and monoclonal antibodies was donated by a local biotech company. Proteins and MAb were dissolved in deionized water.

Columns and Buffer

MAbPac SCX-10, 10 µm, 4 × 250 mm (P/N 074625)

MAbPac SCX-10, 5 µm, 4 × 50 mm (P/N 078656)

CX-1 pH Gradient Buffer Kit (P/N 083274)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System equipped with: SRD-3600 Membrane Degasser, DGP-3600RS Biocompatible Dual-Gradient Rapid Separation Pump, TCC-3000SD Thermostatted Column Compartment with two biocompatible, 10-port valves, WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler, VWD-3400RS UV Detector equipped with a Micro Flow Cell, and a PCM-3000 pH and Conductivity Monitor.

Preparation of Eluents

The CX-1 pH buffer kit consists of 1 bottle of buffer A (pH 5.6) and 1 bottle of buffer B (pH 10.2). Eluent A and B were each prepared by simply diluting the corresponding buffer concentrate 10-fold using deionized water.

Linear pH Gradient Chromatography

The linear pH gradient was generated by running linear gradient from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). For pH gradient analysis carried out on the MAbPac SCX-10, 10 µm, 4 × 250 mm, cation exchange columns, the gradient method in Table 1 was used unless further stated. The UV wavelength was set at 280 nm.

pH Designer

Multi-component Buffer A and buffer B were built using Thermo Scientific™ pH Designer software. Several buffer species were selected for buffer formulation and the pH of the buffer was adjusted with sodium hydroxide *in silico*. The pH elution profile was simulated over 30 min gradient time using linear gradient of 100% Buffer A to 100% Buffer B. The concentration of each buffer species was adjusted to achieve a linear pH elution profile.

Table 1. A 30-min linear gradient method.

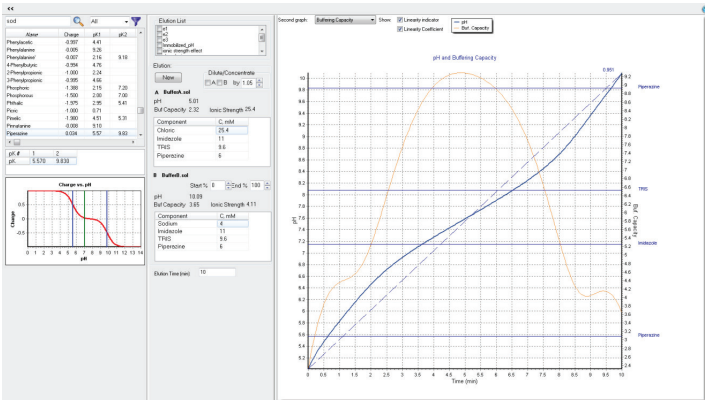
Time (minutes)	Flow rate (mL/min)	%A	%B
0-1	1	100	0
1-31	1	100-0	0-100
31-34	1	0	100
34-40	1	100	0

Results

Buffer Formulation Using pH Designer

Several zwitterionic buffer components with pK_a values between 6 and 10 were selected to build Buffer A and Buffer B. The concentration of all the buffer components were initially set to 10 mM. The pH was adjusted to 5.6 for Buffer A and 10.2 for Buffer B *in silico*. The pH profile was simulated using the built-in elution profile function of pH Designer. The concentration of each component was adjusted until a linear pH profile was achieved, indicated by an R^2 value of 1.0. Figure 1 shows an example of buffer formulation and pH simulation using pH Designer.

FIGURE 1. An example of buffer formulation and simulated pH elution profile using pH Designer.



Linear pH Gradient

Using the formulation determined by the pH Designer software, we made the buffer solutions, conducted the run, and recorded the pH elution profile. Using the gradient method shown in Table 1, six proteins with a range of pI values from 6 to 10 were effectively separated on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. These proteins were lectin (including three isoforms, lectin-1, lectin-2, and lectin-3), trypsinogen, ribonuclease A, and cytochrome C. The chromatogram is shown in Figure 2. The pH value measured in this experiment as a function of time was found to be essentially linear from pH 5.6 to pH 10.2 over a 30 minute period. The correlation coefficient value R^2 was 0.9996. Therefore, the experimental results agreed very well with the simulated one.

An analysis was performed to show that there is a correlation between the elution pH for the peaks and the corresponding pI values of the protein components. Figure 3 is a graph comparing the measured pH values for six protein component peaks in Figure 2 as a function of the corresponding pI values. The measured pH values for the six protein component peaks exhibited a strong linear correlation to the literature based pI values. Thus, after a calibration procedure, this example supports the fact that linear regression coupled with the gradient method described here can be used to estimate the pI of a protein component based on the peak retention time and measured pH.

FIGURE 2. Chromatogram of six proteins separated on a 30-min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. Protein name, retention time, and corresponding pH values are labeled for each protein peak.

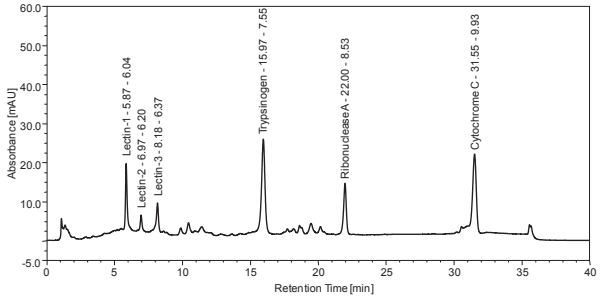
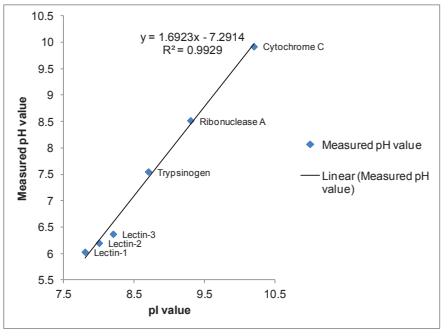


FIGURE 3. A graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 2.

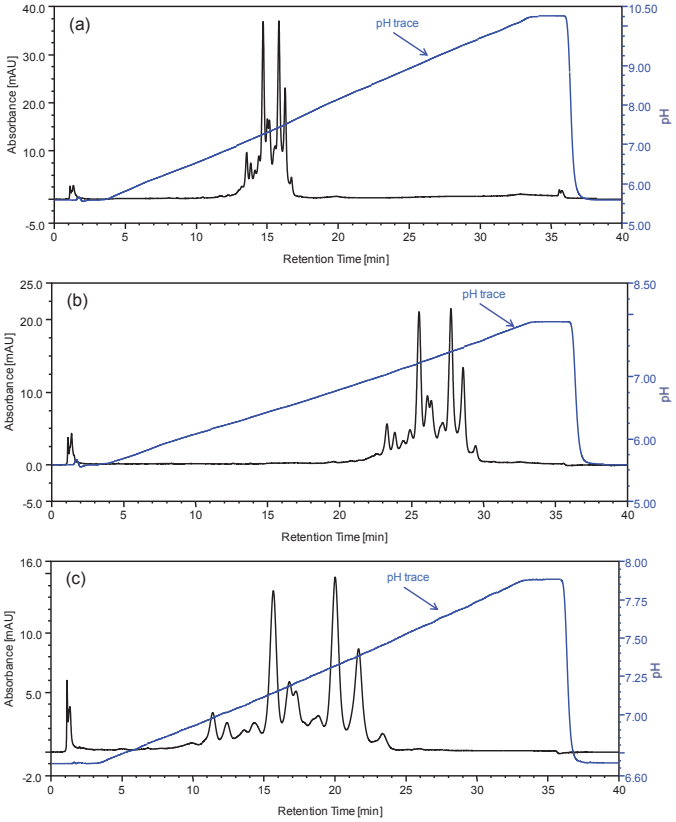


pH Gradient Separation Platform for MAb Variants

Most MAbs have pI values in the range of 6 to 10, so it stands to reason that the pH gradient separation method described in this poster can serve as a platform for charge variant separation. Using a full range of pH gradient from pH 5.6 to pH 10.2, we established the pH elution range in the initial run (Figure 4a) with a pH gradient slope of 0.153 pH unit/min. Further optimization of separation can simply be achieved by running a shallower pH gradient in a narrower pH range. Figure 4b shows the separation profile from pH 5.6 to ph 7.9 with pH gradient slope at 0.076 pH unit/min. Figure 4c shows the separation profile from pH 6.75 to pH 7.9 with pH gradient slope at 0.038 pH unit/min. The pH traces in Figure 4a, 4b, and 4c demonstrated that the pH gradient maintain linear when the slope was reduced to ½ or ¼ of the initial run.

Because the chromatographic profile of the variants were predictable when running a shallower pH gradient. Pump methods for chromatogram shown in Figure 4b and 4c can be automatically generated by writing a post-acquisition script using the MAb variant pH elution range information collected in the initial run (Figure 4a). This example illustrates the advantages of using pH gradient separation platform, which is to simplify and automate the method development for MAb charge variant separation.

FIGURE 4. An example of MAb charge variant separation by linear pH gradient. The separation was carried out on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. (a) Separation by pH gradient, 0% B (pH 5.6) to 100% B (pH 10.2), gradient method was shown in Table 1; (b) Separation by pH gradient, 0% B (pH 5.6) to 50% B (pH 7.9); (c) Separation by pH gradient, 25% B (pH 6.75) to 50% B (pH 7.9).



Conclusions

- A linear pH gradient from pH 5.6 to pH 10.2 was generated using a multi-component zwitterionic buffer system on a cation exchange column.
- A linear pH gradient separation platform enables high resolution, fast and rugged MAb charge variant analysis and automation of method optimization.
- pH Designer Sofware is a powerful research tool for building buffers and predicting pH elution profile.

References

1. Farnan D and Moreno, T., Multiproduct high-resolution monoclonal antibody charge variant separations by pH gradient ion-exchange chromatography. Anal. Chem. 81 (2009) 8846–57.

Australia +61 3 9757 4486
Austria +43 1 333 50 34 0
Belgium +32 53 73 42 41
Brazil +55 11 3731 5140
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