A Novel pH Gradient Separation Platform for Monoclonal Antibody (MAb) Charge-Variant Analysis

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Overview

Purpose: Generate a linear pH gradient for monoclonal antibody (MAb) charge-variant separation.

Methods: Eluent A and eluent B each contains multicomponent 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

Recombinant MAb 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 platform method to accommodate the majority of MAb analyses is desired.

In 2009, Farnan and Moreno reported a method to separate MAb charge variants using pH gradient ion-exchange chromatography.[1] 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.

In this study, we present a novel pH gradient method for cation-exchange chromatography. This method features a multicomponent 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 protein peaks at the time of elution 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. Ruggedness testing of this pH gradient on a Thermo Scientific™ MAbPac™ SCX-10, 5 µm column has shown that peak retention time RSD is less than 0.8% over 300 runs. Extensive testing of the pH gradient method using MAbs and other protein samples has demonstrated the column's capability for fast, high-resolution separations.

Methods

Sample Preparation

All standard proteins were purchased from Sigma. MAbs were a gift from a local biotech company. Proteins and MAbs were dissolved in deionized water.

Columns

MAbPac SCX-10, 10 µm, 4 × 250 mm (P/N 074625)
MAbPac SCX-10, 5 µm, 4 × 50 mm (P/N 078656)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ 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
- PCM-3000 pH and Conductivity Monitor

Preparation of Eluents

Eluent A and B each were prepared by simply diluting the corresponding 10X buffer tenfold using deionized water.

Gradients

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 column, the gradient method in Table 1 was used unless further stated. For pH gradient analysis carried out on the MAbPac SCX-10, 5 µm, 4 × 50 mm column, the gradient method in Table 2 was used unless further stated. Both methods cover the pH range from pH 5.6 to pH 10.2.

TABLE 1. 30-min linear gradient method for the MAbPac SCX-10, 10 µm, 4 × 250 mm cation-exchange column. Total run time is 40 min. The linear pH range covers from pH 5.6 to pH 10.2.

Time (min)	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

TABLE 2. 15-min linear gradient method for MAbPac SCX-10, 5 µm, 4 × 50 mm cation-exchange column. Total run time is 20 min. The linear pH range covers from pH 5.6 to pH 10.2.

Time (min)	Flow Rate (mL/min)	% A	% B
0–1	1	100	0
1–16	1	100–0	0–100
16–17	1	0	100
17–20	1	100	0

Results

The linear pH gradient was achieved by employing a multicomponent buffer system containing multiple zwitterionic buffer species with pI values ranging from 6 to 10. Eluent A was titrated to pH 5.6 and eluent B was titrated to pH 10.2. In this pH range, each buffer species was either neutral or negatively charged. Therefore, they were not retained by the cation-exchange column stationary phase and served as effective buffering agents for the mobile phase and the stationary phase.

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 cytomchrome C. The chromatogram is shown in Figure 1. The pH value measured in this experiment as a function of time was plotted in Figure 2. The pH gradient was essentially linear from pH 5.6 to pH 10.2 over a 30-min period. The correlation coefficient value R^2 was 0.9996.

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 the six protein component peaks in Figure 1 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 1. 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.

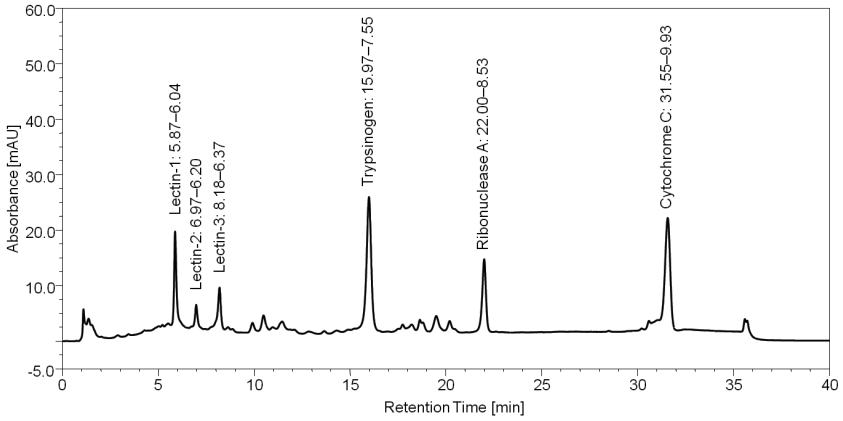


FIGURE 2. A graph showing measured pH values as a function of time. The measured pH values were exported from the same experiment shown in Figure 1. The measured pH values are labeled using blue diamond shape.

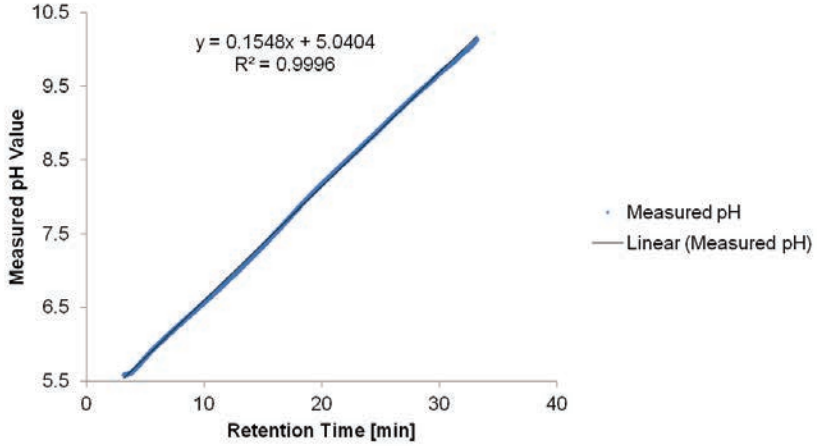


FIGURE 3. A graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 1.

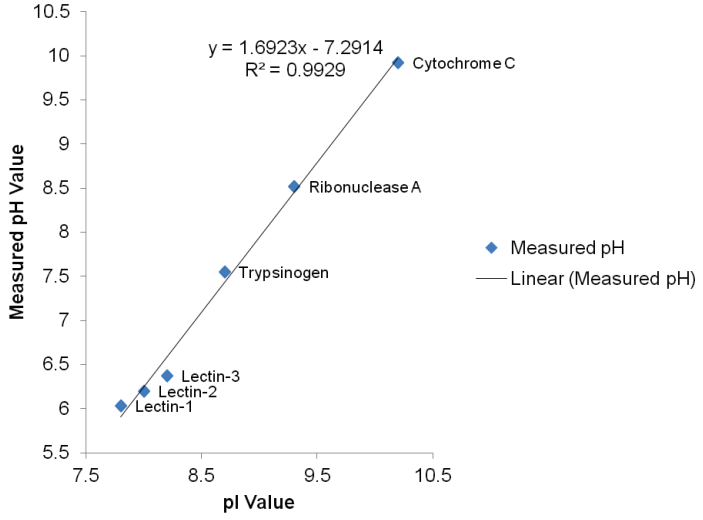


FIGURE 4. An example of MAb charge-variant separation by salt gradient. The separation was carried out on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. Eluent A contained 20 mM MES and 60 mM NaCl (pH 5.6), and eluent B contained 20 mM MES and 300 mM NaCl (pH 5.6). Flow rate was at 0.76 mL/min. A shallow salt gradient was run from 10% B to 30% B from 2 to 32 min, followed by a 2 min wash at 30% B and 1 min wash at 100% B. The column was pre-equilibrated for 15 min at 10% B prior to the gradient. The total run time was 55 min.

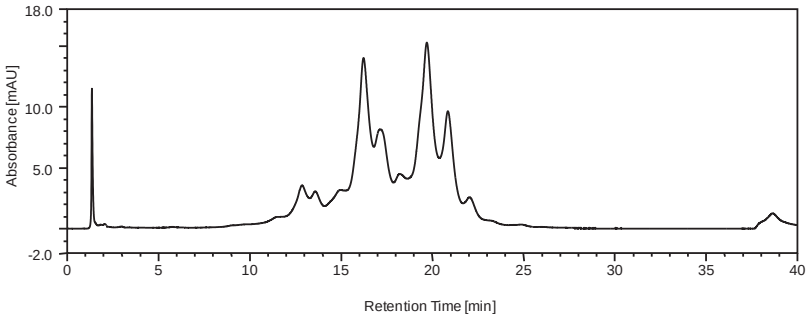
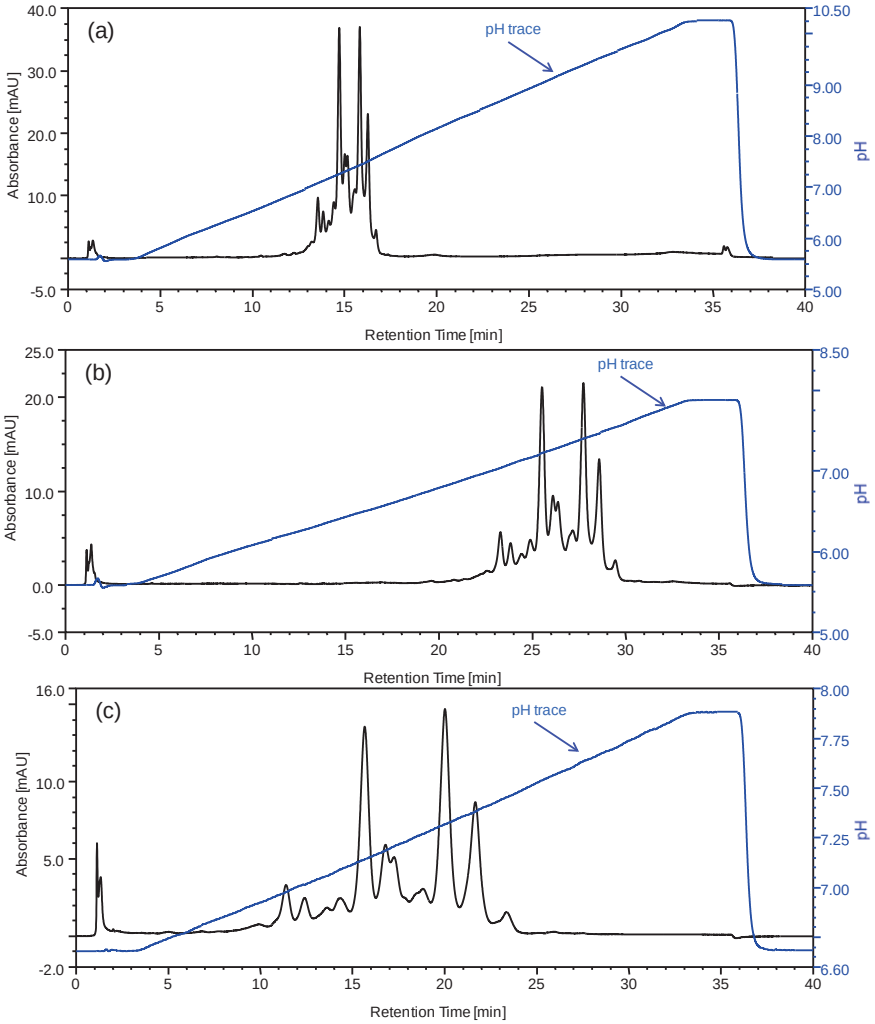


FIGURE 5. An example of MAb charge-variant separation by linear pH gradient. The separation was carried out on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. (a) Separation by pH gradient, 0% B (pH 5.6) to 100% B (pH 10.2), gradient method as shown in Table 1. (b) Separation by pH gradient, 0% B (pH 5.6) to 50% B (pH 7.9). (c) Separation by pH gradient, 25% B (pH 6.75) to 50% B (pH 7.9).

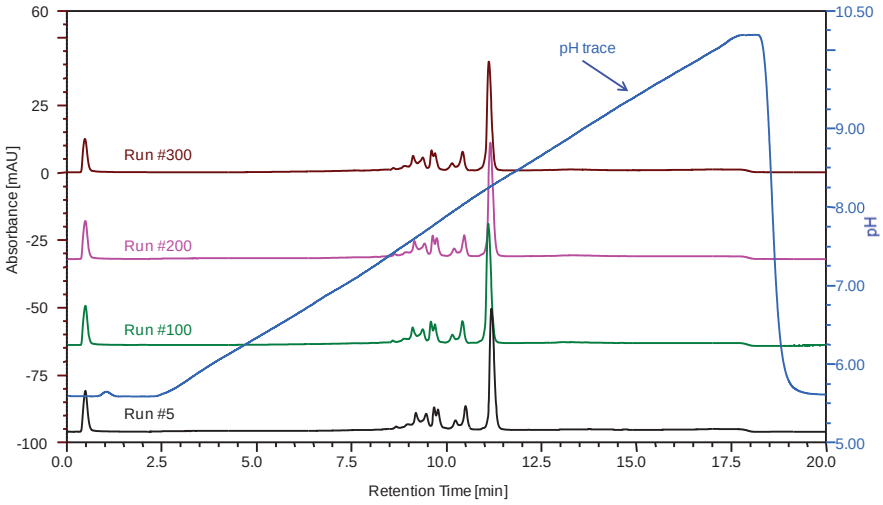


pH Gradient Separation Platform

Most MAbs have pI values in the range of 6 to 10. Our pH gradient separation method can serve as a platform for charge-variant separation. Figure 4 showed the separation of a MAb on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. Using a shallow salt gradient (from 84 mM NaCl to 132 mM NaCl in 30 min), MAb variant peaks were not very sharp, although they were somewhat separated. In order to further optimize the salt method, we may have to experiment with parameters, such as a different buffer salt and a different pH. Instead, we switched to a pH gradient method. 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 5a) 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 5b showed the separation profile from pH 5.6 to ph 7.9, with pH gradient slope at 0.076 pH unit/min. Figure 5c showed 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 5a, 5b, and 5c demonstrated that the pH gradient remained linear when the slope was reduced to ½ or ¼ of the initial run. Therefore, the chromatographic profile of the variants were predictable when running a shallower pH gradient. Pump methods for the chromatograms shown in Figures 5b and 5c can be automatically generated by writing a postacquisition script using the MAb variant pH elution range information collected in the initial run (Figure 5a). This example illustrates the advantages of using a pH gradient separation platform to simplify and automate the method development for MAb charge-variant separation.

In addition, the pH gradient method is fast and rugged. Figure 6 showed a pH gradient run on a MAbPac SCX-10, 5 µm, 4 × 50 mm column. Gradient time was 15 min and the total run time was 20 min. The ribonuclease A peak retention time RSD was less than 0.8% over 300 runs.

FIGURE 6. Ruggedness testing of a pH gradient on a MAbPac SCX-10, 5 µm, 4 × 50 mm column. Gradient method was shown in Table 2. The sample was ribonuclease A.



Conclusion

- A linear pH gradient from pH 5.6 to pH 10.2 was generated using a multicomponent zwitterionic buffer system on a cation-exchange column.
- A linear pH gradient separation platform enables high resolution, fast, and rugged MAb charge-variant analysis.

References

1. Farnan, D.; Moreno, T. Multiproduct High-Resolution Monoclonal Antibody Charge Variant Separations by pH Gradient Ion-Exchange Chromatography. *Anal. Chem.* **2009**, *81*, 8846–57.

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