Development of Ultra-fast pH-Gradient Ion Exchange Chromatography for the Separation of Monoclonal Antibody Charge Variants

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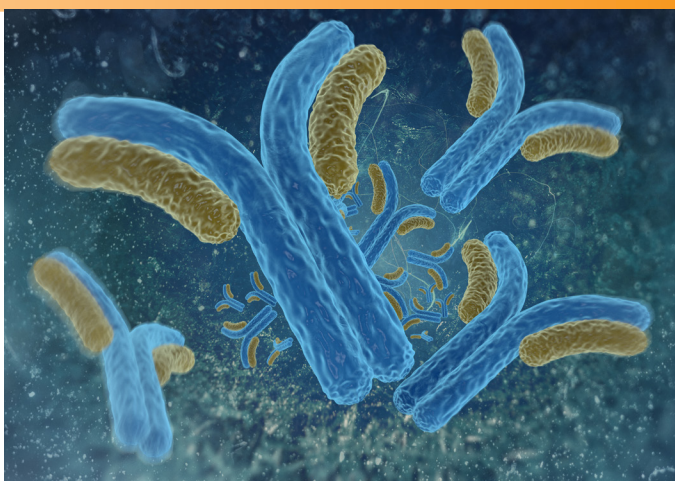
Technical Note 160

Key Words
Critical Quality Attributes, Biotherapeutics, Intact Proteins, Vanquish UHPLC, MAbPac SCX-10 RS Column



Introduction

The pre-formulated buffers for pH gradient, introduced by Thermo Fisher Scientific, have greatly simplified the development of ion exchange chromatography (IEX) of monoclonal antibodies (mAbs). Three features make this simplification possible. The first feature is that the buffers can cover a pH range from 5.6 to 10.2; this pH window enables the characterization of mAbs with a wide range of isoelectric points, providing a global pH gradient ion exchange screening method that will accommodate the majority of therapeutic mAbs. The second feature is that, if a linear solvent gradient is programmed in the pump, the actual pH gradient produced in the column will be linear as well. Although this sounds trivial, scientists involved in pH gradient studies know how difficult it is to develop buffer formulations capable of fulfilling this requirement. The advantage of a genuine linear pH gradient is that the method can be confidently fine-tuned merely by narrowing down the pH range around the mAb and its variants, thus allowing for the adjustment of the gradient time according the resolution requirements. The third feature is the mobile phase preparation: the pre-formulated pH buffers only needs to be diluted by a factor of 10 in deionized water, and the mobile phase is ready to use. Compared to a salt gradient ion exchange chromatography method, where no generic screening can be easily designed, and where method development goes through the rather tedious preparation of several buffers at different pH values, the time and effort invested in method development are substantially reduced.

High resolution pH gradient separations are obtained with 30 minute gradients and relatively long columns, such as the Thermo Scientific™ MAbPac™ SCX-10 column, 10 µm, 4 × 250 mm.[1] Fast separation of mAb variants were demonstrated by using the MAbPac SCX-10 column, 5 µm, 4 × 50 mm with no significant loss in resolution[2] on a Thermo Scientific™ UltiMate™ 3000 BioRS system. In this case, the pH gradient from 5.6 to 10.2 was completed in 7.5 minutes, and the resolution between variants was still satisfactory, despite the short analysis time.

In this work, we show how to push the throughput of pH gradient IEX even further. To achieve this, a MAbPac SCX-10 RS column, 5 µm, 2.1 × 50 mm was operated on a new Thermo Scientific™ Vanquish™ UHPLC system. The Vanquish UHPLC is a fully biocompatible system, suitable for the analysis of intact proteins. The combination of low gradient delay volume and high precision gradient formation makes it the ideal system for high throughput analysis with gradient elution. Here the system was used with the default configuration and a total system gradient delay volume of 175 µL. Fast charge variant separations of 5 mAbs are shown.

Goal

Provide high throughput pH gradient separations of mAb charge variants

Experimental

Vanquish UHPLC, consisting of:

- Vanquish System Base (P/N VH-S01-A)
- Binary Pump H with Default Mixer (P/N VH-P10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)

Chromatographic Conditions	
Column:	MAbPac SCX-10 RS, 5 µm, 2.1 × 50 mm (P/N 082675)
Buffers:	Thermo Scientific CX-1 pH-Gradient buffer A (pH 5.6) 125 mL (P/N 083273) CX-1 pH-Gradient buffer B (pH 10.2) 125 mL (P/N 083275)
Mobile Phase A:	CX-1 pH-Gradient buffer A (pH 5.6) diluted 10x in deionized water
Mobile Phase B:	CX-1 pH-Gradient buffer B (pH 10.2) diluted 10x in deionized water
Column Compartment Temperature	30 °C, forced air
Detector and Conditions	
Detector:	LightPipe™ 10 mm Standard Flowcell (P/N 6083.0100)
Detection Wavelength:	280 nm
Data Acquisition Range:	5 Hz (for flow rate ≤ 0.5 mL/min) and 50 Hz (for flow rate ≥ 1.0 mL/min)
Response Time:	2 s (for flow rates ≤ 0.5 mL/min) and 0.1 s (for flow rates ≥ 1.0 mL/min)
Data Processing	
Software:	Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS)

Samples

mAb Name	Concentration (mg/mL)	Injection Volume (µL)
Bevacizumab	25	1
Cetuximab	5	4
Infliximab	10	4
Trastuzumab	21	2
mAb A	21	2

Results and Discussion

One of the benefits of using CX1 buffers for the pH-gradient is the simplified method development and optimization. This is due to the fact that the pump running a linear solvent gradient will result in a linear pH gradient in the column. This is not the case for most of home-made buffer formulations, which would produce a non-linear pH gradient in response to a linear programmed gradient. A non-linear pH gradient makes method optimization difficult due the uncertainty of the actual effects of any changes in the programmed gradient.

It is recommended to perform a generic screening from pH 5.6 to 10.2 when the pH at which a given mAb elutes is not known. In this work, the generic screening was run in 10 minutes at 0.45 mL/min. As it can be seen in Figure 1 in some cases, satisfactory separation of the charge variants was achieved during the first run. This was the case for cetuximab and infliximab: several charge variants could be resolved with sufficient resolution.

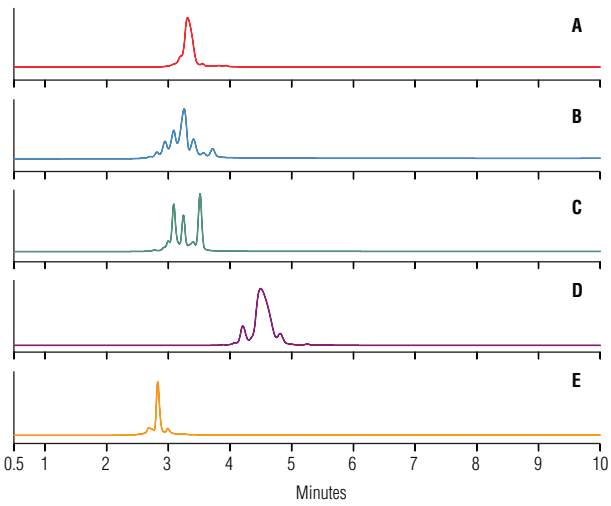


Figure 1. Separation of 5 mAbs with a generic 0→100 %B in 10 min at 0.45 mL/min (method #1 Table 1). Samples are as follows: A) bevacizumab, B) cetuximab, C) infliximab, D) trastuzumab, E) mAb A.

Table 1. Description conditions used for the separation of charge variants of the different monoclonal antibodies.

Method #	Figure	Gradient Range (%B)	Gradient pH Range	Gradient Time (min)	Flow Rate (mL/min)	Gradient Slope* (%B/mL)
1	1	0–100	5.6–10.2	10.0	0.45	22.2
2	2A	20–40	6.5–7.4	5.0	0.50	8.0
3	2B	23–35	6.7–7.2	2.5	1.00	4.8
4	3A	10–35	6.1–7.2	5.0	0.50	10.0
5	3B	10–35	6.1–7.2	2.5	1.00	10.0
6	4A	20–40	6.5–7.4	5.0	0.50	8.0
7	4B	18–27	6.4–6.8	0.8	1.20	9.4
8	5A	35–60	7.2–8.4	5.0	0.50	10.0
9	5B	33–45	7.2–7.7	2.5	1.00	4.8
10	6B	5–30	5.8–7.0	2.5	1.00	10.0

*Slope based on gradient volume

After the generic screening, the method development efforts aimed at decreasing the analysis cycle time and at the same time improving resolution. To achieve this goal, two parameters were modified, namely the pH range and the gradient slope. Here we used gradient slope based on gradient volume, i.e. $\Delta(\%B)/V_G$, where %B is the amount of B eluent and V_G is the volume of mobile phase delivered by the pump during the gradient. A narrower pH window allowed for a reduced run-time, whereas a shallower gradient slope provided better resolution.

The gradient slope of the generic screening between pH 5.6 and 10.2 was 22.2 (%B)/mL; the improved and faster analysis were obtained with gradient slopes of 8 (%B)/mL and 10 (%B)/mL. Details of the conditions are listed in Table 1. Figures 2a, 3a, 4a, and 5a, show the results obtained by this approach. The number of resolved variants was larger than in the initial screening. Gradient time was 5 minutes, hence half of the time of the initial screening.

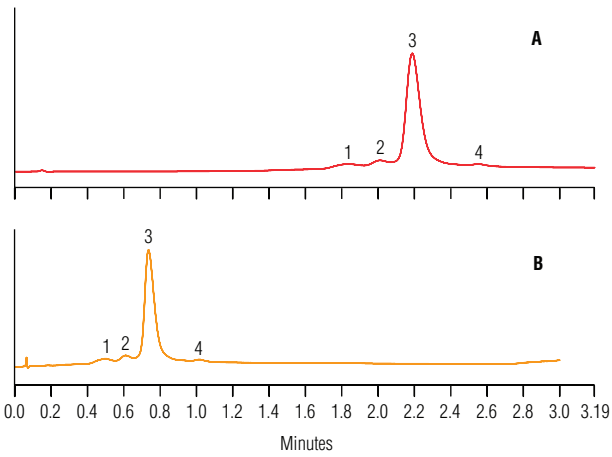


Figure 2. Separation of bevacizumab with different gradient conditions. Chromatogram A: method #2 Table 1; chromatogram B: method#3 Table 1.

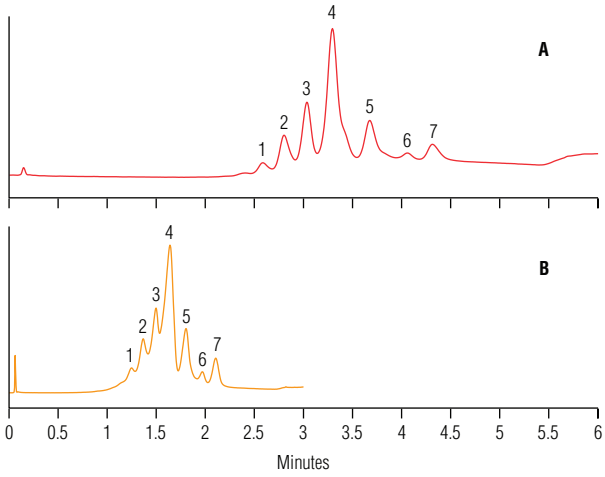


Figure 3. Separation of cetuximab with different gradient conditions. Chromatogram A: method #4 Table 1; chromatogram B: method#5 Table 1.

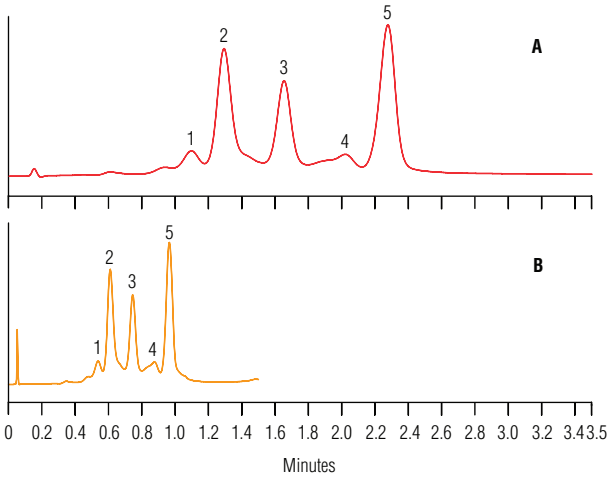


Figure 4. Separation of inflixumab with different gradient conditions. Chromatogram A: method #6 Table 1; chromatogram B: method#7 Table 1.

The following step was used to develop fast analysis cycles compatible with high throughput. With this approach we aimed for a 2.5 minute gradient time or lower, and total analysis time of less than 4 minutes, including column re-equilibration. Data are shown in Figures 2b, 3b, 4b, 5b, and 6b. The purpose was to develop a method suitable for high-throughput that can run at least 300 samples a day. High flow rate was used for this purpose. A high flow rate allows running short gradients with relatively shallow gradient slopes; the shallow slope is required to preserve selectivity between charge variants. Additionally, column equilibration, which is directly dependent on the volume of mobile phase flowing through the column, is reached quicker. MAbPac SCX-10 RS column is pressure rated up to 7000 psi (~ 480 bar), therefore it can be operated at high linear flow rate. In this work, we used flow rates up to 1.2 mL/min.

The chromatographic pattern between methods at moderate and high flow rate was preserved. The separation capabilities of different methods were compared based on the resolution between charge variants. Since in several instances peak pairs were overlapping, and it was not always possible to measure peak width at half height or at the baseline, here we used resolution based on statistical moments. Resolution was calculated directly by Chromeleon 7.2 CDS according to the formula:

$$R = \frac{(t_R^2 - t_R^1)}{2 * (\sqrt{\mu_2^2} + \sqrt{\mu_2^1})}$$

where t_R^2 and t_R^1 are the retention times of the more and less retained peak respectively, and μ_2^2 and μ_2^1 are the related second moment.

In some cases, the ultra-fast separation approach was accompanied by some resolution loss. This is the case of trastuzumab, where average resolution loss was ~ 3%. In the case of the complex variants pattern of cetuximab, the average resolution loss at high flow rate was ~ 13%. Infliximab resolution decreased by ~ 11%, however separation of the 5 main charge variants and 2 minor ones was achieved in 1 minute. This impressive result was obtained by running the column at 1.2 mL/min with a 0.8 min gradient time. In the case of bevacizumab, the ultra-fast separation approach even yielded to ~ 4% improved resolution. The better resolving power can be explained by a slightly narrower pH range and a shallower gradient slope.

mAb A was analyzed only with the generic screening and high flow rate (1 mL/min) method. The high-throughput method provided 19% better resolution on average of 5 charge variants.

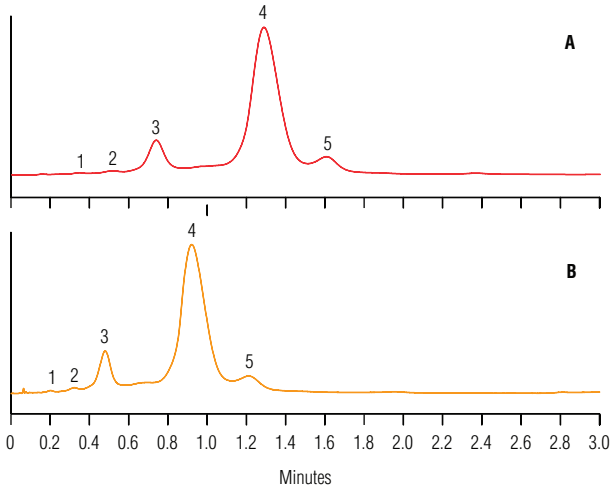


Figure 5. Separation of trastuzumab with different gradient conditions. Chromatogram A: method #8 Table 1; chromatogram B: method#9 Table 1.

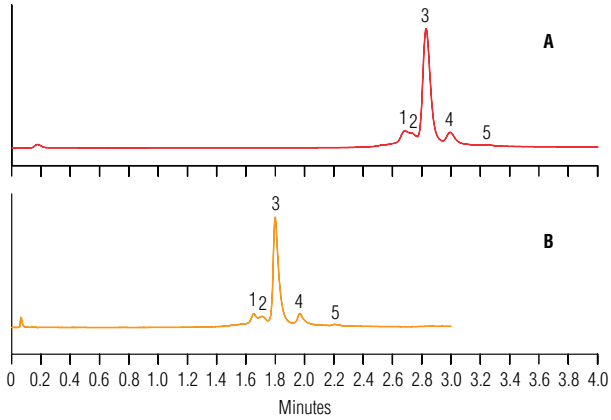


Figure 6. Separation of mAb A with different gradient conditions. Chromatogram A: method #1 Table 1; chromatogram B: method#10 Table 1.

Table 2. Overview of the resolution between charge variants at different conditions. Resolution was calculated by Chromeleon CDS using statistical moments.

Statistical Resolution Charge Variants							
mAb	Gradient Time (min)	Peaks 1–2	Peaks 2–3	Peaks 3–4	Peaks 4–5	Peaks 5–6	Peaks 6–7
Bevacizumab	5	0.94	0.87	1.81	—	—	—
	2.5	1.00	0.89	1.89	—	—	—
Cetuximab	5	0.79	1.14	1.00	1.13	1.28	0.98
	2.5	0.53	1.05	0.94	0.93	1.20	0.91
Infliximab	5	0.68	1.56	1.33	0.87	—	—
	0.8	0.59	1.40	1.30	0.70	—	—
Trastuzumab	5	1.25	1.14	1.70	0.87	—	—
	2.5	1.27	1.11	1.63	0.79	—	—
mAb A	10	0.14	1.31	0.94	1.26	—	—
	2.5	0.32	0.96	1.22	2.02	—	—

Conclusion

The ultra-fast charged variant separations described here are achieved because of several advances in chromatography techniques. The mechanism of pH gradient chromatography lends itself to the use of shorter, faster columns. The availability of high pressure rated small particle size ion exchange columns are a perfect match to pH gradient methodology. The commercial buffer formulations used here form a linear gradient which allows intelligent optimization of the methods. Finally, there is the use of the new Vanquish UHPLC system which has extremely low delay volumes, high precision gradient formation and a totally inert flow path.

Ultra-fast separation that requires total analysis cycle in the order of 2 minutes, including column re-equilibration and injection time, enables users to run more than 1,400 samples during 48 hours continuous operations. To allow unattended tasks with such large amount of samples, the Vanquish UHPLC system can be extended with the Vanquish Charger Module. This can host up to 9000 samples in a thermostatted environment, and transfer them to the Vanquish Autosampler.

References

1. Thermo Fisher Scientific. Thermo Scientific Application Note 20784, *A Novel pH Gradient Separation Platform for Monoclonal Antibody (MAb) Charge Variant Analysis*, AN20784_E, Sunnyvale, CA, **2013**.
2. Thermo Fisher Scientific. Thermo Scientific Application Note 20946, *A Fast and Robust Linear pH Gradient Separation Platform for Monoclonal Antibody (mAb) Charge Variant Analysis*, AN20946_E, Sunnyvale, CA, **2014**.

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