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Application Note

Extended Analysis of mAbs Using Ion-Exchange Chromatography on the Alliance™ iS Bio HPLC System

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Abstract

Large molecule biologics continue to be a major focus in the pharmaceutical market. These biopharmaceutical drugs require rigorous testing and monitoring throughout the production lifecycle beginning with the manufacturing process and ending with lifetime monitoring for stability and efficacy. Ion-exchange chromatography (IEX) is a widely used technique for lysine charge variant monitoring of monoclonal antibodies (mAbs) via high-performance liquid chromatography (HPLC). Ion-exchange methods, which employ either a salt or pH gradient, can be challenging on the chromatographic hardware over time due to the high salt concentration or variable pH used in the mobile phase system.

In this study an Alliance iS Bio HPLC System is employed in a 30-day robustness test using a salt gradient IEX method. A 3-protein standard is used for system baselining each day, while the lysine charge variants of Infliximab are monitored daily. Repeatability of retention time, total area, and relative area are monitored during the study. The chromatographic hardware and column demonstrated consistent performance achieving relative standard deviations as low as 0.1% for retention time, 2.44% for total area, and 0.95% for relative area over the 30 days of testing.

Benefits

- Reproducible multi-day HPLC performance on the Alliance iS Bio HPLC System
- Stable and repeatable salt gradient delivery using quaternary blending
- Consistent results for Infliximab charge variants with sample cooling for biomolecules down to 6 °C

Introduction

Characterization of mAbs is performed using a variety of analytical techniques with the goal of determining similarity between product batches and monitoring for potentially harmful byproducts. During the manufacturing process charge variants may be introduced due to changes in manufacturing conditions, known and/or novel posttranslational modifications, and protein degradation.[1] It is important for manufacturers to monitor for subtle changes in the charge profile of a drug as this can influence stability and efficacy of the final drug product.[2] Charge variant profiling of candidate mAbs is used as an evaluation tool early on in the discovery process. Candidates with charge profiles similar to approved drug products are more likely to exhibit acceptable biological activity profiles.[3]

IEX is a non-denaturing chromatographic method for the separation of charge variants of proteins. This separation technique utilizes charged amino acid side chain residues on the surface of the intact biomolecule and oppositely charged functional groups attached to a stationary phase to separate analytes based on net charge. IEX can be further broken down into sub groups based on the type of charge interaction resulting in analyte separation: cation-exchange chromatography (CEX) where a negatively charge stationary phase binds positively charged analytes, and anion-exchange chromatography (AIEC) where the opposite interaction is the separating factor.[4] CEX is widely used for the separation of mAb charge variants due to the basic isoelectric point of most mAbs, with a salt gradient approach being the most appropriate for separation of acidic and basic variants of the main charge variant peaks.[3,5]

When implementing a high ionic strength method in HPLC, such as a salt-gradient CEX separation, it is important to consider whether the chromatographic equipment to be used is appropriate for the separation. When introduced at high concentrations, salts can precipitate out of solution potentially causing clogging issues

throughout the flow path. Additionally this salting out effect may cause hydrophobic proteins to precipitate out of solution.[3] Mitigation of off-target interactions is another factor to consider when working in the realm of biologics. Large molecule proteins tend to interact with exposed metal surfaces throughout the chromatographic flow path if mobile phase conditions are not ideal due to the very same charged amino acid side chain residues that are used to separate them. This can become difficult when analyzing a mixture of several proteins or a sample containing various isoforms of a protein as each component may have slightly different ideal separation conditions.

The Alliance iS Bio HPLC System has been designed for use with biopharmaceutical applications. (Figure 1) The salty buffers employed in many biopharmaceutical applications can be corrosive when used with traditional stainless steel chromatography systems. The Alliance iS Bio pairs non-ferrous materials such as titanium, PEEK, and MP35N alongside MaxPeak™ High Performance Surface (HPS) Technology in the wetted flow path to ensure robust and rugged operation. In addition, this system utilizes a quaternary blending pump that is ideal for use in IEX chromatography as it allows for the mixing of buffer concentrates to specific separation conditions rather than having to pre-mix mobile phases. This study evaluates the performance of the Alliance iS Bio HPLC System while running a high salt CEX method continuously for 30 days.

Figure 1. The Alliance iS Bio HPLC System.

Experimental

Sample Description

IEX Cation Test Standard (p/n: 186006870 <https://www.waters.com/nextgen/global/shop/standards--reagents/186006870-iex-cation-test-standard.html>) was reconstituted in 500 µL of Mobile Phase A. Contents of the vial were vortexed for 10 seconds to ensure complete reconstitution.

Remicade™ (Infliximab) was diluted to 10 mg/mL in Milli-Q water. 200 µL aliquots were stored at -80 °C until thawed for use. The drug product used in this work was analyzed past expiry.

LC Conditions

LC system:	Alliance iS Bio HPLC System
Detection:	280 nm @ 2 Hz
Vials:	1. Maximum Recovery (p/n: 186005662CV) for IEX Cation Test Standard 2. Total Recovery (p/n: 186005663CV) for Infliximab
Column(s):	BioResolve™ SCX mAb Column, 4.6 x 100 mm, 3 µm (p/n: 186009060)
Column temperature:	30 °C
Sample temperature:	6 °C
Injection volume:	1. 5 µL for IEX Cation Test Standard 2. 10 µL for Infliximab
Flow rate:	0.5 mL/min
Mobile phase A:	100 mM MES Monohydrate
Mobile phase B:	100 mM MES Sodium Salt
Mobile phase C:	1 M Sodium Chloride
Mobile phase D:	Milli-Q Water

Gradient Table

IEX cation test standard					
Time (min)	%A	%B	%C	%D	Curve
0.00	4.6	15.4	5.0	75.0	initial
1.00	4.6	15.4	5.0	75.0	6
31.00	3.8	16.2	30.0	50.0	6
32.00	3.8	16.2	50.0	30.0	6
35.00	3.8	16.2	50.0	30.0	6
35.01	4.6	15.4	5.0	75.0	6
50.00	4.6	15.4	5.0	75.0	6

Infliximab					
Time (min)	%A	%B	%C	%D	Curve
0.00	4.7	15.3	2.5	77.5	initial
1.00	4.7	15.3	2.5	77.5	6
31.00	4.5	15.5	7.5	72.5	6
32.00	3.8	16.2	50.0	30.0	6
35.00	3.8	16.2	50.0	30.0	6
35.01	4.7	15.3	2.5	77.5	6
50.00	4.7	15.3	2.5	77.5	6

Data Management

Chromatography software: Empower™ 3.8.0.1

Results and Discussion

Experimental design for this study focused on continuous use of the system throughout a 30-day period. Sample sets consisted of five replicates of the IEX Cation Test Standard preceded by (10) 30-minute blanks at starting mobile phase condition, three replicates of Infliximab preceded by (10) 30-minute blanks at starting mobile phase

condition, and ending with five more replicates of the IEX Cation Test Standard preceded again by (10) 30-minute blanks at starting mobile phase condition. In total each sample set ran for over 26 hours and were queued to start upon completion of the previous set. Mobile phases, standard, and sample were replaced on an as-needed basis.

When running high salt applications it is critical that the system does not sit for extended periods with salt in the flow path. The application used in this work employs acidic/basic MES buffer concentrates (100 mM) in the A/B lines respectively, and highly concentrated (1 M) NaCl buffer in the C line. These three lines are blended along with water in the D line to a specific pH and NaCl concentration. Up to 500 mM NaCl is used in the chromatographic method at the end of the gradient. Periods of idle flow increase the risk of salt precipitation and can lead to clogging and/or decreased system performance. Over the 30 days of testing the system flow was stopped for minimal time with no system performance issues observed following a stoppage. The ability of the quaternary pump to maintain a consistent flow rate while delivering a precise gradient using salty buffer concentrates speaks to the robustness of the system hardware and is reflected in the consistency of results. The biocompatible design of the Alliance iS Bio HPLC System employs corrosion resistant materials including titanium solvent filters, MP35N tubing, titanium valves, and MaxPeak HPS surface modification throughout the sample flow path. These materials make this system ideal for use under these conditions as long-term exposure of a typical stainless steel HPLC system to a high-salt concentration application would likely result in the issues described above.

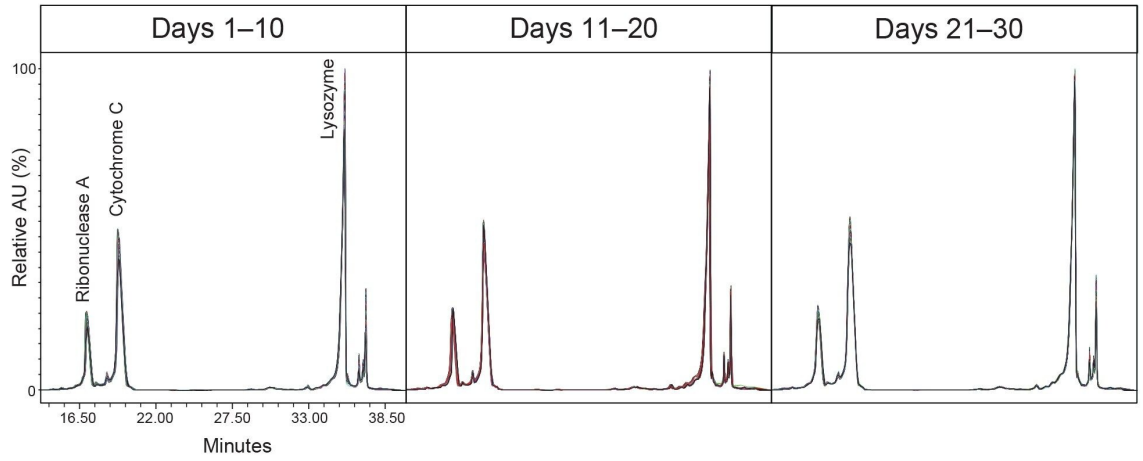

Figure 2. Overlayed chromatograms of IEX Cation Test Standard. Injections are grouped into three buckets: days 1–10 on the left (N=100), days 11–20 in the center (N=84 due to stopping the day 11 sample set before the final standard injections and missing standard vial on day 18), and days 21–30 on the right (N=100). The X-axis of all frames is equal to that shown in the left frame. The Y-axis is scaled to the highest peak in each grouping. The three components in the standard are labeled in the left frame.

Figure 2 displays 10-day grouped overlays of the IEX Cation Test Standard over the 30-days of testing. Chromatographic profiles of the test standard remained consistent over the course of the study. Peak retention time (Figure 3) is consistent throughout the 30-day study. Relative standard deviation (RSD) for retention time, peak area and peak area % are shown in Table I. Retention time RSDs were all well below 1%, which is an excellent indicator of both column and system hardware reproducibility as well as precise gradient delivery reproducibility of the quaternary pump. Small changes in delivery of the quaternary salt gradient can result in large variability in retention times, particularly for quaternary blending. The IEX Cation Test Standard gradient maintains a 20 mM MES buffering system at pH 6.8 while moving through the salt gradient from 50 mM to 300 mM NaCl, before quickly moving to 500 mM NaCl to ensure all sample is removed from the column. The acid/base concentrations are adjusted throughout the salt gradient to maintain a constant pH. Absolute area RSDs below 6% and relative area RSDs below 5% speak to the system and column performance overall, but particularly highlight robustness and reproducibility of the Alliance iS Bio autosampler hardware.

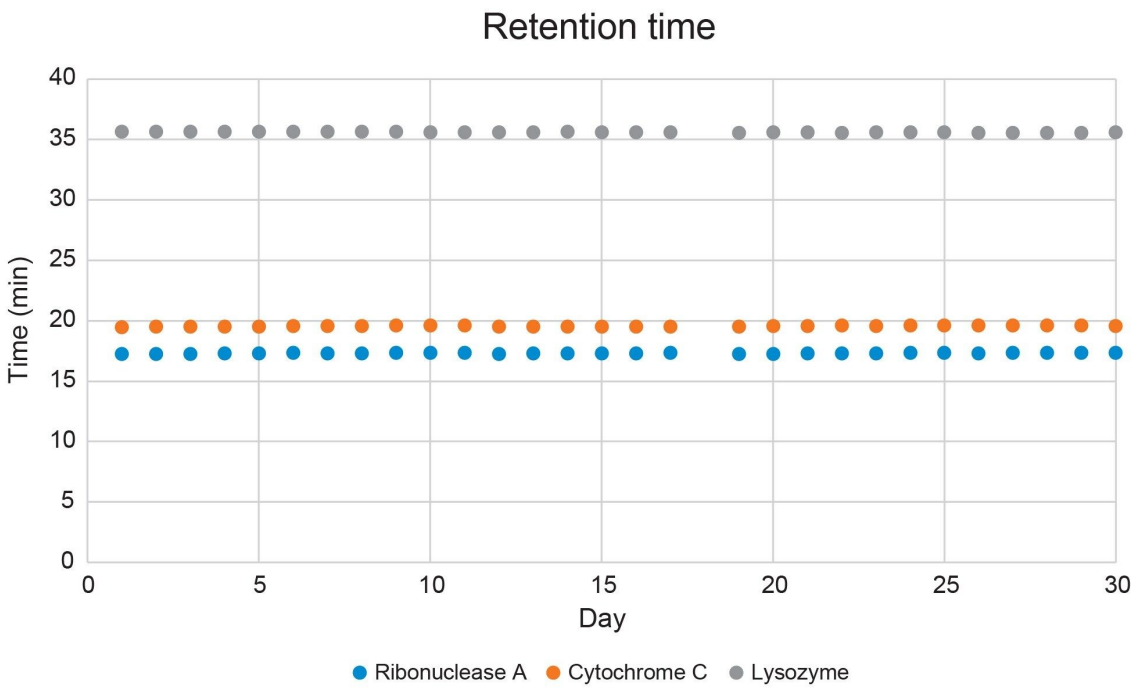

Figure 3. Retention time averages of IEX Cation Test standard from each day of testing. N=10 in all cases except day 11 (N=5) where the sample set was stopped before the final replicates of standard were injected, day 18 (N=0) due to an empty vial, and day 19 (N=9) where the 1st injection was missed. Error bars are present (though too small to see) and represent a single standard deviation from the average.

In addition to the system performance metrics observed using the IEX Cation Test Standard an Infliximab sample was tested daily to monitor for changes in lysine peak relative areas or amounts of acidic and basic variants in the sample. This sample is a good measure of overall system performance as the lack of resolution between the acidic/basic variants and the main charge peaks means that any degradation in sample quality or system performance can result in large variations in relative areas. The salt gradient used for this sample is shallower than that of the test standard, moving from 25 mM to 50 mM NaCl over the course of 30 minutes, translating to changes in the C line of 0.17%/minute. The pump hardware was able to reproducibly deliver these small changes in the gradient over the course of the study.

Relative standard deviation			
Measure	Component		
	Ribonuclease A	Cytochrome C	Lysozyme
Retention time (min)	0.20	0.24	0.10
Area (AU)	5.72	2.44	3.98
% Area	4.06	2.05	1.88

Table 1. Retention time, total area, and relative area relative standard deviation measurements for each of the three components in the IEX Cation Test Standard over the course of the 30 day study. N=289 in all cases.

Figure 4 displays 10-day grouped overlays of Infliximab over the 30-days of testing. As with the test standard, chromatographic profiles of the Infliximab charge variants remain reproducible throughout this study and maintain consistent relative areas. (Table II) Relative area %RSDs for the three main lysine charge variant peaks were all below 1.5% over 30 days. Of particular importance, the acidic and basic variants of the main peaks also remained highly reproducible, achieving relative area %RSDs below 6.0%. As previously stated, sample and standard were changed on an as-needed basis. 500 µL aliquots of Infliximab were used in this study and with 30 µL total being injected per sample set, this means that the Infliximab sample was held in the sample manager for well over ten days before being replaced. The Alliance iS demonstrated excellent sample temperature control over this period keeping sample and standard chilled to 6 °C, helping to prevent sample degradation which is reflected in the stability of each component.

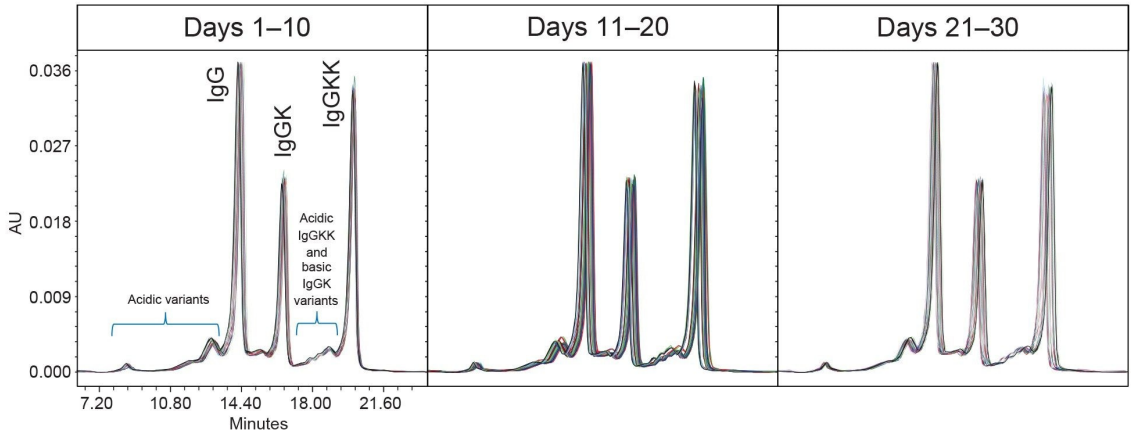

Figure 4. Overlayed chromatograms of Infliximab. Injections are grouped into three buckets: days 1–10 on the left (N=30), days 11–20 in the center (N=27 due to missing sample vial on day 18), and days 21–30 on the right (N=30). The X-axis of all frames is equal to that shown in the left frame. The Y-axis is scaled to the highest peak in each grouping. Components in the sample are labeled in the left frame. IgG=Immunoglobulin G. IgGK=Immunoglobulin G with an additional lysine. IgGKK=Immunoglobulin G with two additional lysine.

As demonstrated using the IEX test standard, retention times for the major lysine charge variant peaks of Infliximab are highly consistent throughout this study with %RSDs below 1.5%.

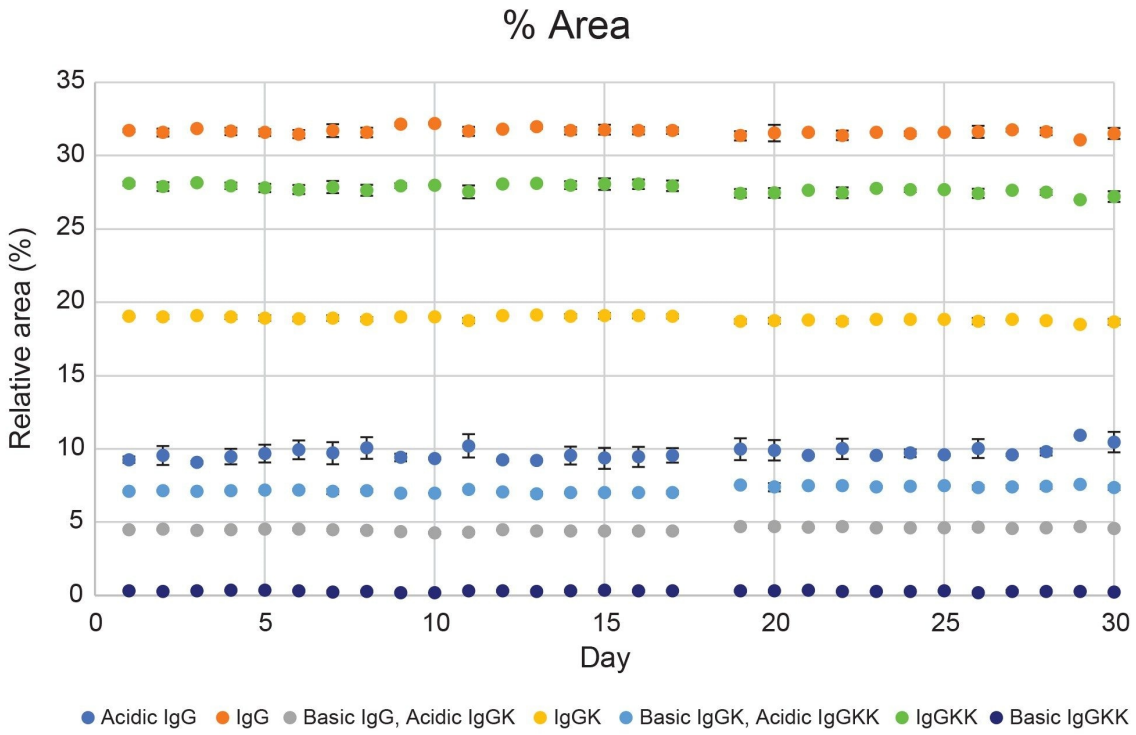

Figure 5. Relative area averages of Infliximab lysine charge variants over 30 days of testing. N=3 in all cases except day 18 where N=0 due to an empty vial. Error bars represent a single standard deviation from the average. IgG=Immunoglobulin G. IgGK=Immunoglobulin G with an additional lysine. IgGKK=Immunoglobulin G with 2 additional lysine.

Relative standard deviation						
Measure	Component					
	Acidic IgG	IgG	Basic IgG/ Acidic IgGK	IgGK	Basic IgGK/ Acidic IgGKK	IgGKK
Retention time (min)	N/A	1.19	N/A	1.03	N/A	1.07
% Area	5.93	0.95	1.35	1.00	2.92	1.28

Table 2. Retention time and relative area relative standard deviation measurements for each component in the Infliximab separation over the course of the 30 day study. N=87 in all cases. Acidic and basic variant retention time results are not shown as they are a combination of multiple peaks in the chromatogram.

Conclusion

When running a chromatographic system continuously for 30 days, it would not be unexpected to see a drop-off in performance, particularly when running a high salt application. This study investigated the Alliance iS Bio HPLC System for a high-use scenario employing a CEX salt gradient for the separation of lysine charge variants in a mAb. The method used in this study was a complex quaternary blending method using concentrated salty buffers which can lead to system degradation over time. The Alliance iS Bio System employs biocompatible materials such as MP35N and titanium throughout the wetted flow path to prevent corrosion when used in salty bio-applications. Additionally, the system provided rugged long-term sample manager temperature control limiting sample degradation. The Alliance iS Bio HPLC System demonstrated excellent robustness while maintaining system performance, delivering a reproducible gradient, and reducing system downtime. Over the course of 30 days, an IEX Cation Test Standard consisting of three proteins achieved retention time, total area, and relative area %RSDs of less than 0.24%, 5.72%, and 4.06% respectively. The mAb sample (Infliximab) maintained consistent relative areas over the course of the study for both the high-abundance lysine charge variant peaks (%RSD ≤1.28% in all cases) as well as the low-abundance acidic and basic charge variants (%RSD ≤5.93% in all cases). Overall, the system and column demonstrated consistent chromatographic performance over the entire 30-day period.

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