Analysis of Therapeutic Monoclonal Antibodies Using Volatile pH Gradient Cation Exchange Chromatography Directly Coupled to Native Mass Spectrometry

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ABSTRACT

Purpose: To optimize a volatile pH gradient mobile phase system for native mass spectrometry analysis of monoclonal antibody charge variants using weak cation exchange (WCX) chromatography.

Methods: Weak cation exchange chromatography using volatile pH gradient directly coupled to the Orbitrap mass spectrometer for charge variant analysis of four therapeutic monoclonal antibodies (Vedolizumab, Secukinumab, Pertuzumab and Trastuzumab).

Results: Volatile pH gradient buffers separated multiple charge variants of four mAbs with different pI values. Lysine variants were separated from the main product peak in the WCX separation and glycosylation forms of different lysine variant were identified using ReSpect deconvolution and Sliding Window analysis in BioPharma Finder 3.1. In addition, deamidation was detected on Secukinumab.

INTRODUCTION

Therapeutic monoclonal antibody (mAb) products are highly heterogeneous due to the post translational modifications and other modifications that occur during manufacturing and storage. These modifications often result in charge variants which can be effectively separated using cation exchange chromatography (CEX). Salt gradient and pH gradient methods have been used for charge variant analyses of proteins including monoclonal antibodies.[1,2] Conventional salt and pH gradient methods cannot be directly coupled to mass spectrometry due to high concentration of buffer salt and the non-volatile nature of the mobile phases. It is desirable to create a wide range of volatile pH gradient, enabling direct detection of mAb charge variants by native mass spectrometry. In this work, we present an optimized CEX–MS method for profiling of intact mAbs.

MATERIALS AND METHODS

Sample Preparation

Vedolizumab, Secukinumab, Pertuzumab and Trastuzumab samples were provided by pharmaceutical companies. All samples were buffer exchanged into 50 mM ammonium acetate using Micro Bio-Spin™ 6 columns (BioRad). Final concentration of the samples were approximately 5-15 µg/µL.

LC-MS Methods

Samples were separated by a Thermo Scientific™ ProPac™ Elite WCX (weak cation exchange) column (2 x 50 mm, 5 µm particles) on a Thermo Scientific™ Vanquish™ F UHPLC system at a flow rate of 400 µL/min. After the UV detection, the flow was split to obtain 100 µL/min flow rate going into a Thermo Scientific™ Q Exactive™ UHMR Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. The mobile phase system used for all LC separations consisted of 25 mM ammonium bicarbonate and 30 mM acetic acid as buffer A (pH 5.21) and 10 mM ammonium hydroxide in 2 mM acetic acid as buffer B (pH 10.18). Gradient methods used for each mAb are shown in Table 1. Column compartment was held at 25 ºC, and UV was monitored at 280 nm. Sample load was 60 µg for all mAbs except pertuzumab (120 µg). MS parameters are shown in Table 2.

Table 1. Gradient methods used for separation of mAb charge variants

Vedolizumab				Secukinumab				Pertuzumab and Trastuzumab			
Time (min)	%A	%B	Curve	Time (min)	%A	%B	Curve	Time (min)	%A	%B	Curve
0.0	60	40	5	0.0	60	40	5	0.0	60	40	5
0.5	60	40	5	0.5	60	45	5	0.5	60	40	5
10.0	50	50	7	10.0	50	50	7	10.0	20	80	5
10.1	0	100	5	10.1	0	100	5	10.1	0	100	5
12.0	0	100	5	12.0	0	100	5	12.0	0	100	5
12.2	60	40	5	12.2	60	40	5	12.2	60	40	5
22.0	60	40	5	22.0	60	40	5	22.0	60	40	5

Table 2. MS parameters

Mass range	Spray voltage	Sheath gas	Auxiliary gas	Capillary temp.	S-lens level
4,000 – 10,000	3.8 kV	40	10	275 ºC	200
In-source CID	**Microscans**	**AGC target**	**Maximum IT**	**Resolution**	**Probe temp.**
100 eV	10	3×10^6	200 ms	12500 or 25000	150

Data Analysis

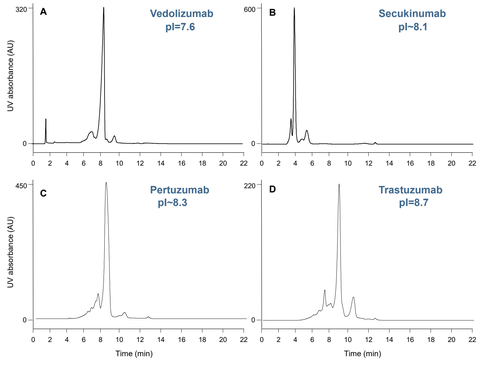
Data were analyzed with Thermo Scientific™ BioPharma Finder™ 3.1 software. WCX-MS spectra were deconvoluted using ReSpect algorithm and Sliding Window analysis.

RESULTS

Volatile pH Gradient Buffers for WCX-MS

Cation exchange chromatography has been widely used to analyze charge variants of mAbs. Elution of the sample can be achieved by increasing the salt concentration of the mobile phase or applying a pH gradient that changes the charge of the protein from positive to neutral when the mobile phase pH equals the pI of the protein. Traditionally both elution modes utilize non-volatile salts or buffering species that cannot be directly coupled to mass spectrometry due to severe ion suppression. Recently Florian Fussl et al have reported a set of volatile pH gradient mobile phases that can be used for direct coupling of CEX and MS.[1] This buffer system is comprised of ammonium bicarbonate and ammonium acetate. Due to the lack of buffering effect around pH 7 and 8 they employed shallow and/or curved gradients for mAbs that elute in this pH range. A short strong cation exchange chromatography column was used for this study to limit the column buffering effect. Here we have applied the same mobile phase system with a short weak cation exchange column. Despite the buffering effect of the weak cation exchange functional groups (carboxylate), separation of four mAbs with different pI values were achieved demonstrating the applicability of this buffer system to weak cation exchange chromatography (Figure 1). Gradient method was optimized for mAbs with different pI values. When shallow gradient was applied, it was important to start with a lower %B than where the separation occurs. This is to prevent the steep pH increase while loading the sample.

Figure 1. Separation of charge variants of four mAbs on ProPac Elite WCX column using volatile pH buffers; (A) Vedolizumab (B) Secukinumab (C) Pertuzumab (D) Trastuzumab



Charge variant CEX-native mass spec analysis

Separation of different charge variants in the LC using CEX enables simpler and improved annotation of glycoforms for each charge variant peak that would normally co-elute in reversed phase or size exclusion chromatography. The most commonly reported charge variants separated by CEX are C-terminal lysine truncation, deamidation of glutamine, pyroglutamate formation on the N-terminal glutamine and succinimide formation of aspartic acid residues[2]. Some of these modifications are critical to the safety and efficacy of the drug while others serve as probes to understand the consistency of the product.

All the mass spectra were deconvoluted and analyzed by BioPharma Finder using ReSpect algorithm and Sliding Window. As shown in Figure 2, Trastuzumab chromatogram has several charge variant peaks with a main peak at 8.5 min. The large basic peak that elutes after the main peak around 11 min was identified to be a variant with one unclipped C-terminal lysine.

For both of these peaks, six main glycoforms–G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, G0F/G0, G0/G0 as expected were observed in MS (Figure 2, C). For pertuzumab, close analog of trastuzumab, similar separation was achieved as shown in Figure 1, C. Overall 29 glycoforms were assigned with less then 10 ppm of mass accuracy, using the BioPharma Finder identification option including some low abundant sialylated forms (Figure 3).

Figure 2. WCX-UV-MS analysis of Trastuzumab (A) UV trace (B) TIC chromatogram (C,D) MS spectra of 2 charge variants.

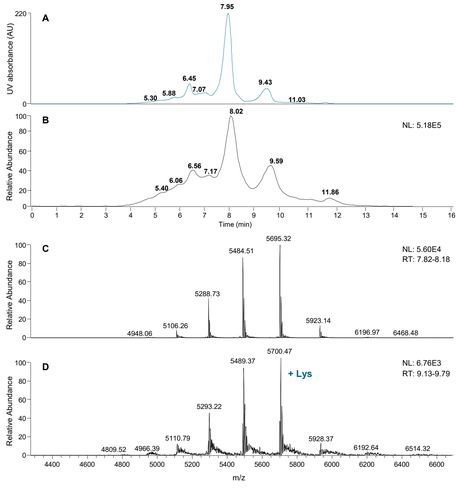
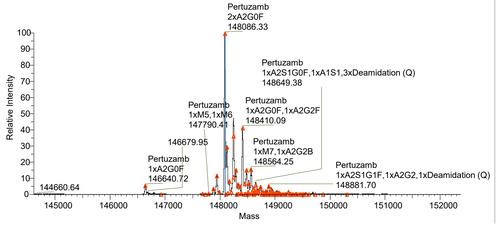
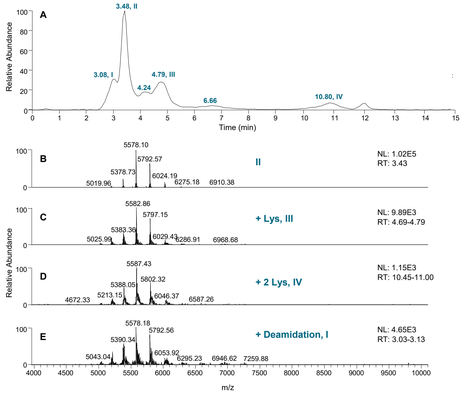


Figure 3. BioPharma Finder 3.1 deconvolution and identification results of WCX-MS analysis of Pertuzumab at resolution of 25,000@ 400 m/z.



Secukinumab has lower predicted pI than Trastuzumab and Pertuzumab (Figure 1) and for its charge variant analysis we used slightly modified gradient (Table 1). Figure 4 shows results of CEX-native MS analysis of Secukinumab. Besides Lys unclipped isoforms we also identified multiple deamidation isoforms (Figure 4, E). Deamidation results in a very small change in mass (0.98 Da) which makes it very difficult to resolve in MS unless there is chromatographic separation before MS injection that shifts retention time forward.

Figure 4. WCX-UV-MS analysis of Secukinumab (A) TIC chromatogram (B-E) MS spectra of different isoforms.



Analysis of Vedolizumab

Out of four analyzed mAbs, Vedolizumab has lowest pI due to N-terminal pyroglutamate in heavy chains. Glutamine conversion to pyroglutamate decreases pI from 5.65 to 0.94. Additionally it has slightly different distribution of main glycoforms (Figure 6). However we still achieved a good separation using same buffers but optimized gradient (Table 1; Figure 5). When a 100% A to 100% B gradient is run with this volatile pH gradient, there is a steep increase in pH around pH 7 and pH 8. A shallow and curved gradient was used to compensate for the rapid increase in pH. This optimization is critical for low pI mAbs such as Vedolizumab. The separation obtained using this optimized gradient was comparable to the non-volatile linear pH gradient result as shown in Figure 5.

Figure 5. Comparison of Vedolizumab separation using non-volatile linear pH gradient buffers and volatile pH gradient buffers (A) Thermo Scientific™ CX-1 pH gradient buffers (B) Volatile pH gradient buffers

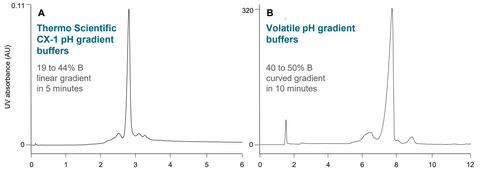
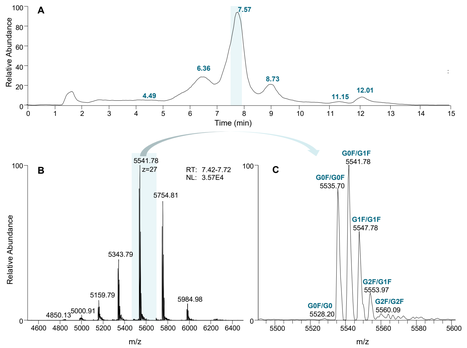


Figure 6. WCX-UV-MS analysis of Vedolizumab (A) TIC chromatogram (B) MS spectra of main peak (C) Distribution of main glycoforms, charge 27+



CONCLUSIONS

A set of volatile mobile phases which can generate a pH gradient from 5.21 to 10.18 were used to separate multiple charge variants of four different therapeutic mAbs on a WCX column. Direct coupling of WCX to MS using these mobile phases resulted in simpler and improved annotation of multiple charge variants with high mass accuracy. This study demonstrates the power of CEX-MS using pH gradient for characterization of intact mAbs.

- Volatile pH gradient buffers formulated using ammonium bicarbonate and ammonium acetate successfully separated multiple charge variants on a WCX column and are compatible with MS
- WCX-MS method allowed the detection of different glycoforms for each separated charge variant
- A deamidated form of secukinumab eluted earlier than the main peak and was successfully annotated using the BioPharma Finder software
- Using curve gradient, Vedolizumab, which has lower pI value than other mAbs, was separated into several charge variants

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2. Bailey AO, Han G, Phung W, Gazis P, Sutton J, Josephs JL, Sandoval W., Charge variant native mass spectrometry benefits mass precision and dynamic range of monoclonal antibody intact mass analysis. MAbs. 2018 Nov-Dec;10(8):1214-1225.

TRADEMARKS/LICENSING

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