

## Charge-Variant Analysis of Antibody-Drug Conjugates with Cation-Exchange Chromatography

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This is an Application Brief and does not contain a detailed Experimental section.

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### Abstract

This application brief demonstrates charge-variant analysis of antibody-drug conjugates with salt and pH gradient methods on BioResolve SCX mAb Columns.

### Benefits

Demonstrating the utility of BioResolve SCX mAb Columns for successful charge-variant analysis of ADCs

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### Introduction

#### Background

Antibody-drug conjugates (ADCs) are a promising type of biotherapeutic in oncology because of improved drug delivery efficiency and substantially reduced side effects compared to traditional chemotherapy.

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A cytotoxic small molecule drug can be delivered to tumor cells via covalent linkage to a tumor antigen specific monoclonal antibody. Amino groups of lysine residues or sulfhydryl groups of interchain cysteine residues are suitable conjugation sites on mAbs, as is the case for ado-trastuzumab emtansine (Kadcyla) and brentuximab vedotin (Adcetris), respectively. ADCs are challenging to characterize due to their structural complexity, and only a few applications of ion-exchange chromatography on the analysis of ADCs have been reported.<sup>1</sup> Recently, a new ion-exchange chromatography technology, the BioResolve SCX mAb Column, was developed for robust charge-variant analysis of mAbs. In this work, we expand upon the capabilities of this technology and demonstrate charge-variant analysis of two ADCs, lysine-conjugated ado-trastuzumab emtansine and a cysteine-conjugated ADC.

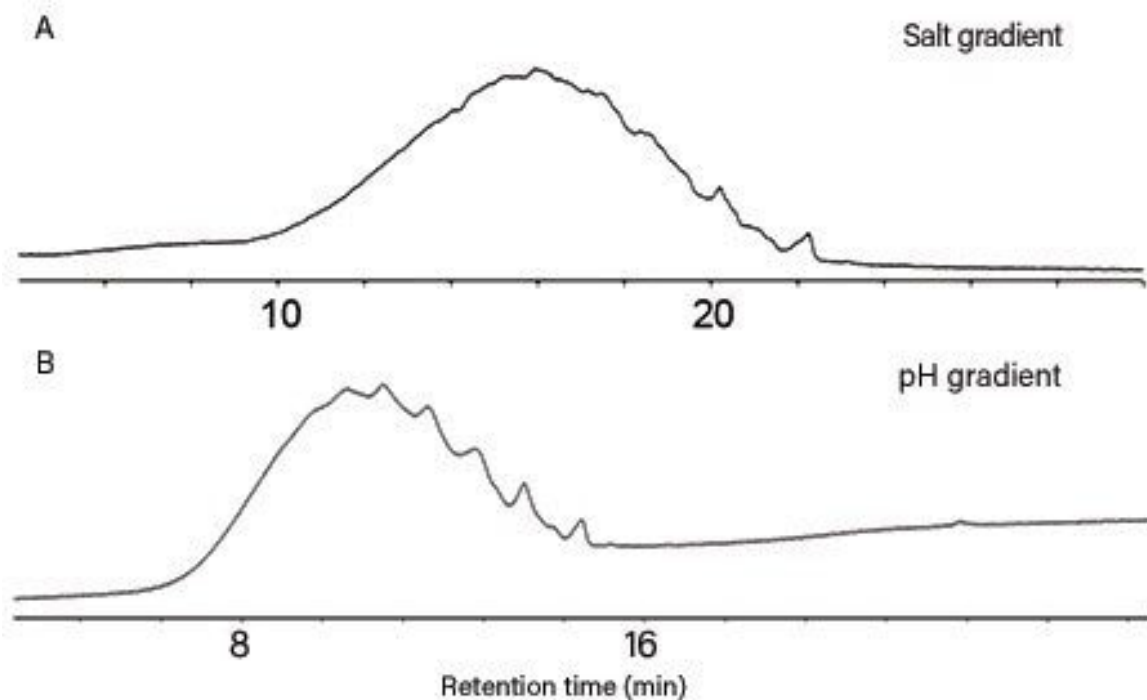


Figure 1. Charge-variant profile of ado-trastuzumab emtansine obtained with ion-exchange chromatography. (A) UV chromatograms obtained with a salt gradient method of 20 mM MES (pH 6.6) with a linear increase of sodium chloride concentration from 30–110 mM in 30 minutes at 0.80 mL/min on a BioResolve SCX mAb, 4.6 x 100 mm Column. (B) UV chromatograms obtained with pH gradient methods on a BioResolve SCX mAb 4.6 x 50 mm Column using BioResolve CX pH Concentrates, mobile phases, and a linear increase of mobile phase B percentage from 0–100% in 30 minutes at 1.00 mL/min. UV detection was at 280 nm.

## Results and Discussion

In ado-trastuzumab emtansine, uncharged cytotoxic agent DM1 is covalently linked to lysine residues on humanized monoclonal antibody trastuzumab via a neutral linker. That is, where there is a conjugated residue, a basic lysine amino moiety has been exchanged for neutral amide group. With 90 possible conjugation sites in trastuzumab, it would be predicted that the charge-variant profile of ado-trastuzumab emtansine is highly heterogenous. Since each addition of drug load decreases the net positive charge by one, the charge-variant

profile of ado-trastuzumab emtansine is indicative of drug load and drug distribution. However, high heterogeneity and increased hydrophobicity from drug conjugation<sup>2</sup> make it a challenging analyte for charge variant analysis.

Method optimization was first performed for the separation of ado-trastuzumab emtansine using a salt gradient. In all the tested conditions, 20 mM MES pH 6.6 and a gradient from 30–110 mM sodium chloride in 30 minutes at 0.80 mL/min gave the most optimized resolution on a BioResolve SCX mAb, 4.6 × 100 mm Column (Figure 1A). For another attempt at method optimization, a newly developed pH-gradient mobile-phase system, based on BioResolve CX pH Concentrates, was employed.<sup>3</sup> Using a generic gradient of 0-100% mobile phase B in 30 minutes and a flow rate of 1.00 mL/min on a BioResolve SCX mAb, 4.6 × 50 mm Column, a similar peak profile was obtained (Figure 1B). Nevertheless, slightly improved resolution was observed with the generic pH gradient method. This separation provides an indication of ado-trastuzumab emtansine's overall extent of modification, as can be extrapolated from how far shifted the peak profile is versus native, unmodified trastuzumab (Figure 2A).

For many analysts, there is a concern that the hydrophobic payloads of ADCs will exacerbate secondary interactions with chromatographic stationary phases. Where these concerns exist, organic co-solvent can be used to attenuate any potential hydrophobic interactions but attention must be made to not denature the protein analyte. To test the extent of secondary interactions between ADC analytes and the BioResolve SCX mAb Column, different percentages of acetonitrile were blended into the pH gradient mobile phases using a quaternary solvent manager. Slightly higher retention times and lower peak areas of ado-trastuzumab emtansine were observed in the UV chromatograms with 5% and 10% acetonitrile (Figure 2B), demonstrating that there was little to no secondary interaction in the fully aqueous separation. Meanwhile, the slight increases in retention time were probably a result of slightly reduced eluotropic strengths from the acetonitrile dilution. Comparable results were observed with salt gradient separations.<sup>4</sup>

Similar experiments were performed on a discontinued, cysteine-linked ADC from Pfizer (Figure 3). Unlike lysine-linked ADCs, a cysteine linked ADC would be predicted to exhibit less heterogeneity. First and foremost, the conjugation involves far fewer potential sites of modification. In terms of electrostatic effects, the mAb conjugation sites undergo a net neutral modification, though the linker payload itself can introduce a charge. Regardless, more interpretable charge-variant profiles can generally be obtained. With method optimization, 20 mM MES pH 5.4 and a salt gradient from 140–220 mM sodium chloride in 30 minutes at 0.80 mL/min was found to yield reasonable resolution on a BioResolve SCX mAb Column. With the BioResolve CX pH Concentrates and their corresponding mobile-phase system used with a generic gradient and flow rate of 1.00 mL/min, a similar peak profile was observed. Of note, the acidic shoulder of the first main peak was, however, better resolved with

the pH gradient method. The addition of 5% acetonitrile to the pH gradient mobile phases resulted in a similar UV peak area, which again demonstrates there to be little to no secondary interactions between the ADC and BioResolve SCX mAb stationary phase. Separately, the effects of adding 10% acetonitrile to the mobile phase was investigated. With this experiment, a dramatic change was observed in the peak profile, which suggests that the use of 10% acetonitrile can lead to the dissociation of light and heavy chains in the ADC, and that it is too denaturing to be suitable for use in this application.

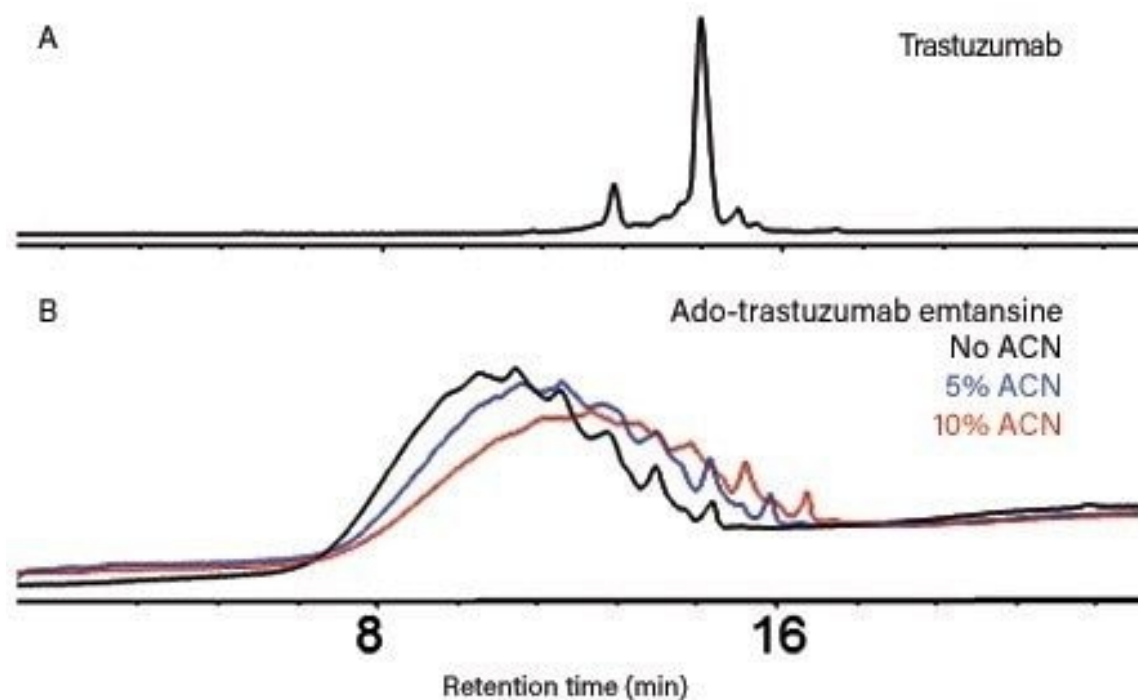


Figure 2. Charge-variant profile of trastuzumab and ado-trastuzumab emtansine obtained with pH gradient ion-exchange chromatography. UV chromatograms of trastuzumab (A) and ado-trastuzumab emtansine (B, black trace) obtained on a BioResolve SCX mAb, 4.6 x 50 mm Column using BioResolve CX pH Concentrates and a linear increase of mobile phase B percentage from 0 to 100% in 30 minutes at 1.00 mL/min. (B) Overlay of UV chromatograms obtained with pH gradient methods on a BioResolve SCX mAb, 4.6 x 50 mm Column using BioResolve CX pH Concentrates and mobile phases without, with 5%, and with 10% acetonitrile. UV detection was at 280 nm.

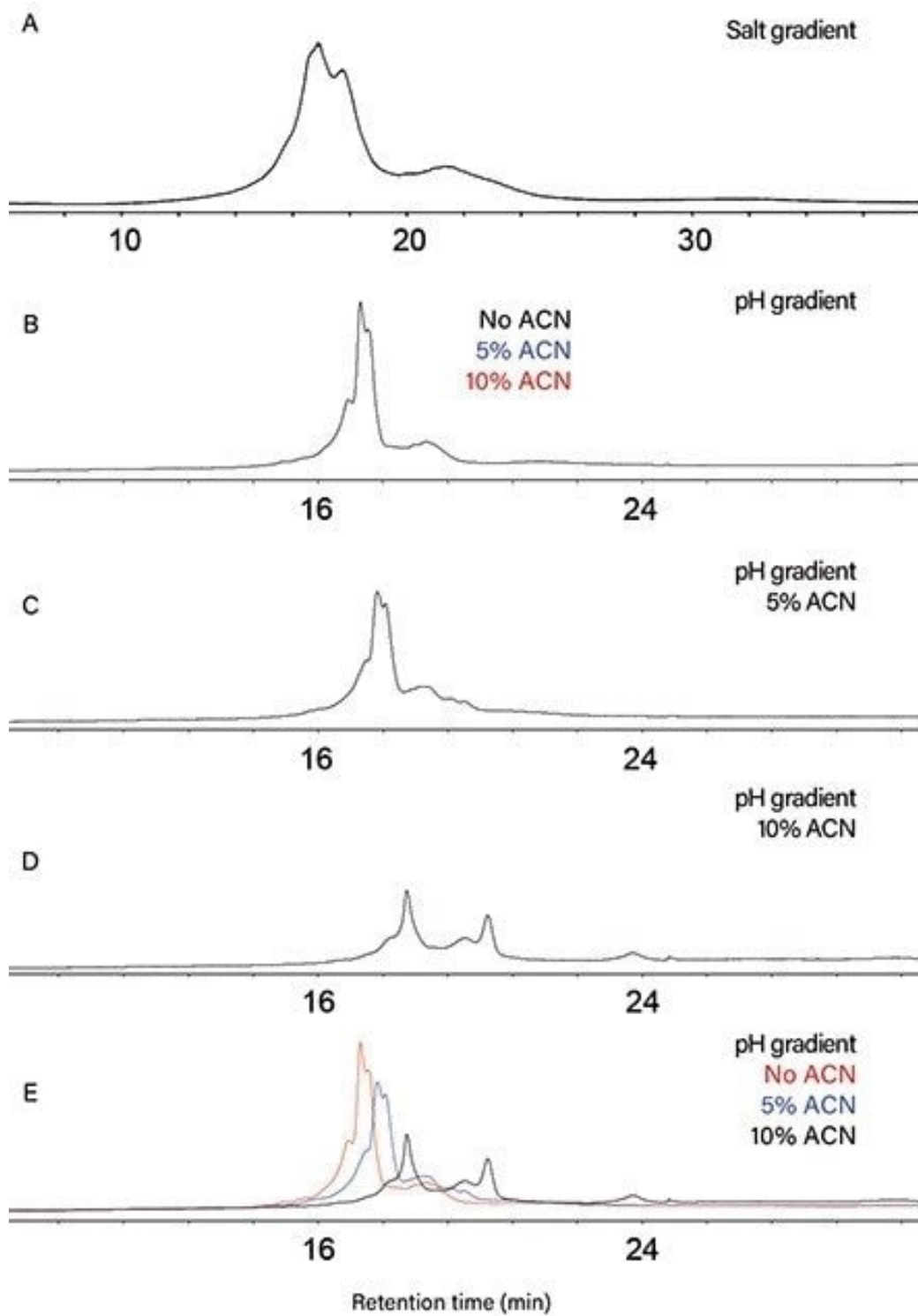


Figure 3. Charge-variant profile of a discontinued, cysteine-linked antibody drug conjugate from

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*Pfizer as obtained with ion-exchange chromatography. (A) UV chromatograms obtained with a salt gradient method of 20 mM MES (pH 5.4) and a linear increase of sodium chloride concentration from 140–220 mM in 30 minutes at 0.80 mL/min on a BioResolve SCX mAb, 4.6 x 100 mm Column. UV chromatograms obtained with pH gradient methods on a BioResolve SCX mAb, 4.6 x 50 mm Column using BioResolve CX pH Concentrates and mobile phases without (B), with 5% (C), or with 10% acetonitrile (D) and a linear increase of mobile phase B percentage from 0–100% in 30 minutes at 1.00 mL/min. (E) Overlay of UV chromatograms obtained with pH gradient methods on a BioResolve SCX mAb, 4.6 x 50 mm Column using BioResolve CX pH Concentrates and mobile phases without, with 5%, and with 10% acetonitrile. UV detection was at 280 nm.*

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## Conclusion

The capability of the BioResolve SCX mAb Column for charge-variant analysis of ADCs was demonstrated on a lysine- and cysteine-linked ADC using both salt and pH gradient elution mechanisms. Robust separations were achieved with optimized salt gradient methods and a generic pH gradient method using the BioResolve CX pH Concentrates. Little to no secondary interactions were observed between the ADCs and BioResolve SCX mAb Column stationary phase as shown with experiments entailing the addition of organic co-solvent into the mobile phase. In sum, it has been demonstrated that BioResolve SCX mAb Columns, as well as the BioResolve CX pH Concentrates, have utility in charge-variant analyses of ADCs. In addition to these examples of 1D-LC analysis, the merit of these separations should also be judged by their potential usefulness as the first dimension in a multidimensional separation, where their analytical power could be further amplified.

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## References

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### Available for purchase online

BioResolve SCX mAb Column, 3  $\mu$ m, 4.6 mm x 100 mm, with mAb Charge Variant Standard <  
<https://www.waters.com/waters/partDetail.htm?partNumber=176004349>>

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