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POSTER NOTE

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Separation of Bispecific mAbs Using Hydrophobic Interaction Chromatography (HIC)

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ABSTRACT

Purpose: Demonstrate separation of a bispecific mAb from related species generated during the assembly using the Thermo Scientific™ MAbPac™ HIC-20 column.

Methods: Various chromatography methods including size exclusion chromatography (SEC), ion-exchange chromatography (IEC) using pH gradient, reversed-phase (RP) chromatography and hydrophobic interaction chromatography (HIC) were investigated for the separation of four species that are generated during bispecific mAb assembly. For HIC, three different column chemistries were tested using ammonium sulfate and sodium phosphate mobile phases.

Results: Among chromatography methods that were investigated, HIC using MAbPac HIC-20 column provided the best separation of all four species generated during the bispecific mAb assembly.

INTRODUCTION

Advancement of recombinant antibody technologies has enabled the development of many different types of monoclonal antibody (mAb) therapeutics such as antibody-drug conjugates (ADC) and bispecific mAbs.[1] Bispecific mAbs have specificity towards two different antigens which may be utilized to recruit killer T cells to the tumor cell or antagonize two different tumor specific antigens simultaneously.[2] Bispecific mAbs are engineered with two different mAbs that have different antigen binding sites. Therefore it is important to separate and monitor possible mis-assembled mAb biproducts that are produced during the process. Chromatographic separation of multiple species formed during assembly of a bispecific mAb could be challenging due to structural similarities between parental antibody domains and the desired bispecific mAb product. In this study, we have investigated various chromatography methods including size exclusion chromatography (SEC), ion-exchange chromatography (IEC), reversed-phase (RP) chromatography and hydrophobic interaction chromatography (HIC) to separate and analyze four related species that are generated during assembly of a bispecific mAb.

MATERIALS AND METHODS

Sample Preparation

The bispecific mAb samples were donated by a biotech company.

Columns

MAbPac SEC-1, 5 µm, 4.0 × 300 mm (P/N 074696)
MAbPac SCX-10, 10 µm, 4.6 × 250 mm (P/N 074625)
MAbPac RP, 4 µm, 3.0 × 50 mm (P/N 088645)
MAbPac HIC-10, 5 µm, 4.6 × 100 mm (P/N 088480)
MAbPac HIC-20, 5 µm, 4.6 × 100 mm (P/N 088553)
MAbPac HIC-Butyl, 5 µm, 4.6 × 100 mm (P/N 088558)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:

- SR-3000 Solvent Rack (P/N 5035.9200)
- LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)
- WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- VWD-3400RS Rapid Separation Variable Wavelength Detector (VWD) equipped with micro flow cell (P/N 5074.0010)
- Chromatography was controlled by Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System.

Chromatography Conditions

Mobile phases are shown in Table 1. Flow rate, gradient and column temperature are shown on the right side of each figure.

Table 1. Mobile Phases Used for Various Chromatography Methods

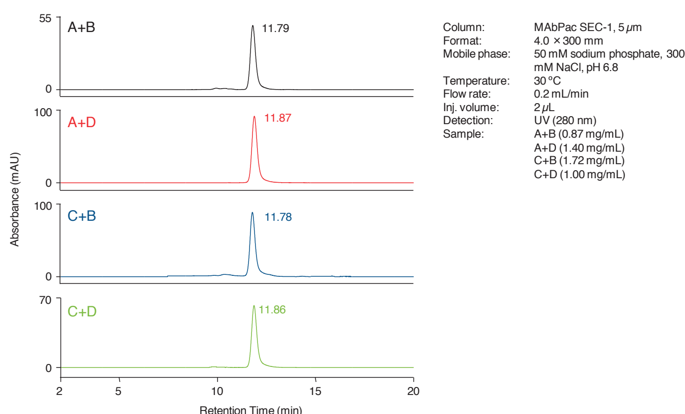
	Mobile phase A	Mobile phase B
MAbPac SEC-1	50 mM sodium phosphate, 300 mM NaCl, pH 6.8	
MAbPac SCX-10	CX-1 pH Gradient Buffer A (P/N 085346)	CX-1 pH Gradient Buffer B (P/N 085348)
MAbPac RP	H_2O/TFA (99.9:0.1 v/v)	MeCN/H_2O/TFA (90:9.9:0.1 v/v/v)
MAbPac HIC-10	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0	100 mM sodium phosphate, pH 7.0
MAbPac HIC-20 (a)	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0	100 mM sodium phosphate, pH 7.0
MAbPac HIC-20 (b)	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)	50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)
MAbPac HIC-Butyl	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0	100 mM sodium phosphate, pH 7.0

RESULTS

SEC Anlaysis of Bispecific mAbs

A bispecific mAb was generated using four domains, which resulted in four possible combinations of the domains. To obtain pure form of the product it is essential to separate these bi-products from the desired species. First, to determine whether these species can be separated based on size, SEC was evaluated. A MAbPac SEC-1 column with sodium phosphate mobile phase was used to analyze four species. As indicated by the retention time of the four species, no separation was observed which implies minimal differences in size of these species.

Figure 1. SEC separation of four species generated during bispecific mAb assembly



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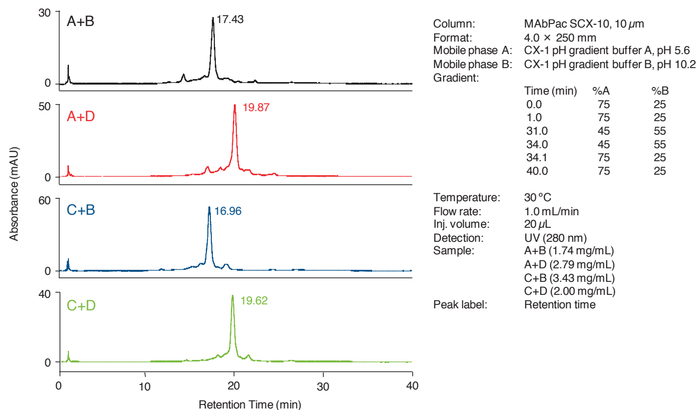
IEC Analysis of Bispecific mAbs Using a Linear pH Gradient Method

Next, IEC using CX-1 pH Gradient Buffers was evaluated for the separation of four bispecific mAb species. After running all samples from 0 to 100% Buffer B, the gradient was optimized to 25 to 55% B for maximum separation. The four species were separated into two groups. A+B and C+B eluted earlier with retention times of 17.42 and 16.96 and A+D and C+D were eluted later with retention times of 19.87 and 19.62. Domains B and D determined the retention behavior of these species.

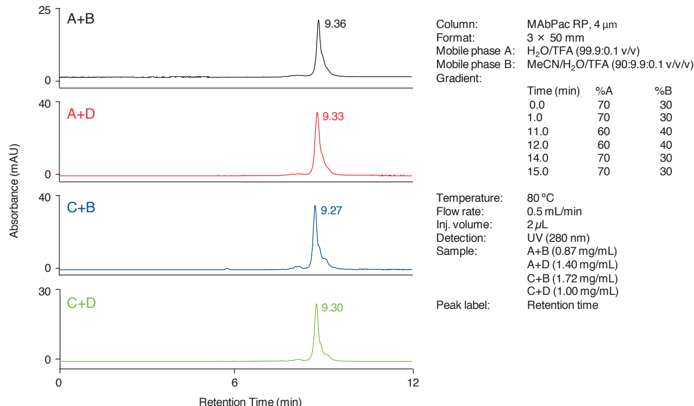
Figure 2. pH Gradient separation of four species generated during bispecific mAb assembly



RP Analysis of Bispecific mAbs

In order to evaluate separation of four bispecific mAb species using differences in hydrophobic interaction with the stationary phase, reversed-phase chromatography and HIC were performed. Figure 3 shows RP chromatograms using standard water to acetonitrile gradient with trifluoroacetic acid as the ion-pair reagent. Even with a shallow gradient (30 to 40% mobile phase B), the retention time of all four species were within 0.10 minute.

Figure 3. RP separation of four species generated during bispecific mAb assembly



HIC Analysis of Bispecific mAbs

Column chemistry could significantly affect the selectivity and/or the resolution of analytes. Here three HIC columns with different ligand chemistries-polyamide, amide and butyl-were evaluated (Figure 4, 5 & 6). All three HIC columns showed same order of elution; 1) C+B 2) A+B 3) C+D 4) A+D. However best resolution was achieved using the MAbPac HIC-20 column with an ammonium sulfate gradient from 0.8 to 2 M. Addition of isopropanol to the mobile phases A and B resulted in a separation pattern similar to the IEC using pH gradients which the separation of A+B and C+D was larger but the separation between C+B and A+B and separation between C+D and A+D were significantly reduced (chromatogram not shown; Table 2). Retention times obtained from all the chromatography modes and columns are shown in Table 2. In addition, the retention time differences between C+B and A+B, A+B and C+D and C+D and A+D were calculated for all the chromatograms. Although IEC using pH gradient buffers provided the best separation between A+B and C+D, MAbPac HIC-20 column gave the best separation between all four species.

Figure 4. Separation of four species generated during bispecific mAb assembly on MAbPac HIC-10

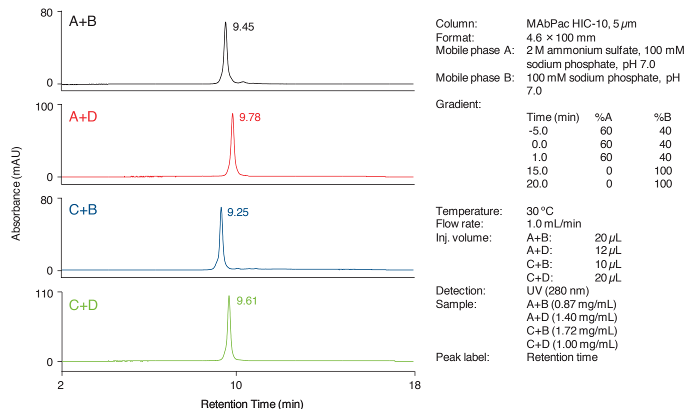


Figure 5. Separation of four species generated during bispecific mAb assembly on MAbPac HIC-20

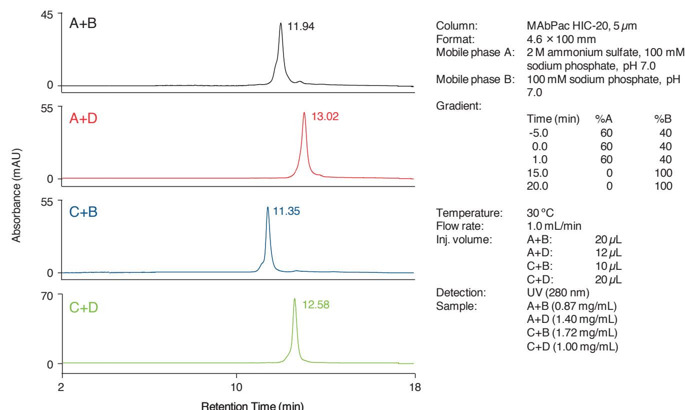


Figure 6. Separation of four species generated during bispecific mAb assembly on MAbPac HIC-Butyl

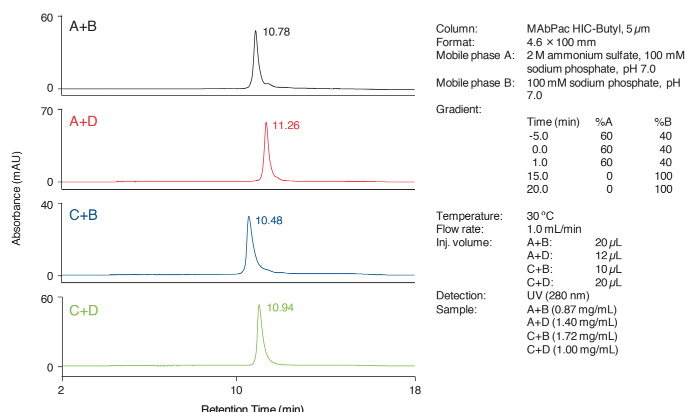


Table 2. Retention times and retention time differences of four species generated during bispecific mAb assembly on MAbPac columns

	A+B	A+D	C+B	C+D	Δ[(A+B)-(C+B)]	Δ[(C+D)-(A+B)]	Δ[(A+D)-(C+D)]
MAbPac SEC-1	11.79	11.87	11.78	11.86	**0.01**	**0.07**	**0.01**
MAbPac SCX-10, CX-1 pH Gradient Buffers	17.43	19.87	16.96	19.62	**0.47**	**2.20**	**0.24**
MAbPac RP	9.36	9.33	9.27	9.30	**0.10**	**-0.06**	**0.02**
MAbPac HIC-10	9.45	9.78	9.25	9.61	**0.20**	**0.16**	**0.17**
MAbPac HIC-20 (a)	11.94	13.02	11.35	12.58	**0.59**	**0.64**	**0.44**
MAbPac HIC-20 (b)	7.44	8.42	7.25	8.29	**0.19**	**0.85**	**0.13**
MAbPac HIC-Butyl	10.78	11.26	10.48	10.94	**0.30**	**0.16**	**0.32**

CONCLUSIONS

- For separations of structurally related species generated during assembly of bispecific mAbs, screening of multiple chromatographic methods including SEC, IEC, HIC, and RP, is necessary.
- Among chromatography methods that were investigated, HIC using MAbPac HIC-20 column provided baseline separation of all four species generated during the bispecific mAb assembly.

REFERENCES

1.Kontermann, R. & Brinkmann, U. *Drug Discovery Today* 20, 838-847 (2015).

2.Kontermann, R. *MAbs* 4, 182–197 (2012).

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