Poster Note 72100

HPAE-FLD Method for Separation of Human α1 Acid-Glycoprotein and Bovine Fetuin 2-Aminobenzamide (2AB)-Labeled Oligosaccharides

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ABSTRACT

Purpose: To demonstrate the HPAE-FLD method described in proposed USP <212> to separate 2-AB-labeled *N*-linked oligosaccharides from human α1 acid-glycoprotein and bovine fetuin

Methods: Oligosaccharides released from two glycoproteins are labeled with a fluorophore followed by their purification and HPAE-FLD analysis. Two different methods for purification of the labeled oligosaccharides from unbound labeling reagent were tested along with two different elution conditions.

Results: The data show that both the variations tested here do not significantly affect the retention time profiles. And that the method described here is suitable for routine labeling and profiling of oligosaccharides released from glycoproteins.

INTRODUCTION

Glycosylation plays an important role in protein structure and function. It is a post-translational modification that requires interaction of various enzymes and substrates[1]. Various factors that affect protein glycosylation, such as cell type, cell age, cell culture conditions, etc., can lead to significant batch-to-batch variability. Product quality control of glycoprotein therapeutics is, therefore, important. This has resulted in an increased demand for methods to characterize these carbohydrates.

High-Performance Anion-Exchange (HPAE) chromatography is used to separate anionic analytes, such as carbohydrates, that are or can be ionized at high pH values (>pH 12). HPAE uses hydroxide-based eluents at high pH to produce anions from analytes that would not be anionic at neutral pH.

HPAE combined with fluorescence detection (HPAE-FLD) offers an effective tool for selective and sensitive analysis of *N*-linked glycans released from glycoprotein therapeutics and labeled with the fluorophore 2-AB. In this work, an HPAE-FLD method for analyzing *N*-linked glycans that is described in proposed USP General Chapter <212>[2] is used to separate the 2-AB labeled *N*-linked glycans from human α1 acid-glycoprotein and bovine fetuin.

This method is easily executed on a Thermo Scientific™ Dionex™ ICS-5000+ system with an FLD detector. If the Dionex ICS-5000+ is equipped with the dual pump and an electrochemical detector (ED), one half of the system can be used for this method while the other half is used for traditional HPAE-PAD, including separation of the unlabeled, released glycans, monosaccharide analysis, & sialic acid analysis. HPAE combined with pulsed amperometric detection (HPAE-PAD) is widely used for carbohydrates[3].

MATERIALS AND METHODS

1. **Sample preparation:** This consisted of two steps as described below.
   a) **Sample Labeling:** Oligosaccharides released from the two proteins used in this study, human α1 acid-glycoprotein and bovine fetuin, were labeled using a Signal 2-AB labeling kit (Prozyme)
   b) **Removal of free 2-AB:**
   Two methods were used for removal of unbound labeling reagent:
      i. Method 1: Using size exclusion (SEC) spin column
      ii. Method 2: Using a solid phase extraction (SPE) cartridge
2. **Chromatographic Analysis**: For sample volume, column, & detection conditions refer to Figure 4.
   Two different elution methods were tested. The gradient time programs for both methods are as shown below in Tables1 and 2.

Table 1. Unmodified Elution.

Time (min)A	Solution A (%)	Solution B (%)	Solution C (%)	Elution
0	80	10	10	Initial condition
15	80	10	10	50 to 150 mM NaOAc, isocratic 0.05 N NaOH
70	60	30	10	150 to 450 mM NaOAc, isocratic 0.05 N NaOH
94	0	90	10	NaOAc wash, no gradient
99	0	90	10	
105	0	10	90	NaOH wash, no gradient
110	0	10	90	
111	80	10	10	Re-equilibrium
130	80	10	10	

Table 2. Modified Elution.

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)	Elution
0	80	10	10	Initial condition
15	80	10	10	50 to 150 mM NaOAc, isocratic 0.05 N NaOH
70	62	30	8	150 to 450 mM NaOAc, isocratic 0.05 N NaOH
94	8.9	90	1.1	NaOAc wash, no gradient
99	8.9	90	1.1	
105	0	10	90	NaOH wash, no gradient
110	0	10	90	
111	80	10	10	Re-equilibrium
130	80	10	10	

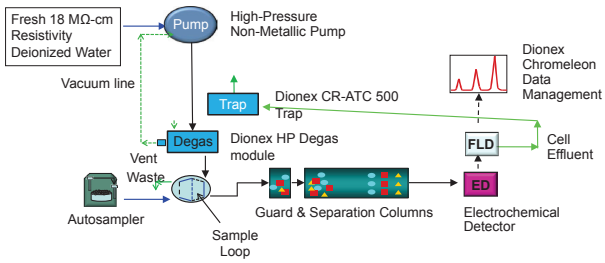
3. **Instrument:** Dionex ICS 5000+ HPIC system (Figure 1A) hyphenated to Thermo Scientific™ Dionex™ UltiMate™ 3000 RS Fluorescence Detector. (Figure 1B). Figure 2 shows the flow diagram.

Figure 1. (A) Dionex ICS-5000⁺ HPIC system (B) Dionex UltiMate 3000 RS Fluorescence Detector.

A.

B.

Figure 2. Flow Diagram for the Dionex ICS-5000⁺ System Configured for ED and FLD Detections.



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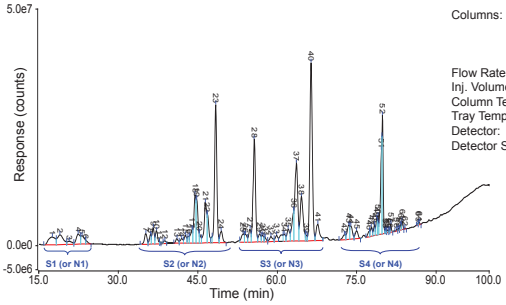
RESULTS

This work describes a method for qualitative analysis of protein glycosylation through profiling of glycans released through enzymatic hydrolysis. The method involves labeling the released oligosaccharides with a fluorescent dye, 2-AB, followed by chromatographic analysis using HPAE-FLD. The goal of this work is to demonstrate that the procedural variations tested in these two steps do not significantly affect the elution behavior of labeled oligosaccharides.

The labeled oligosaccharides are purified from the unbound dye using either of two methods: 1) SEC or 2) SPE. The two methods used for purification of labeled oligosaccharides from unbound labeling reagent do not change their elution profile.

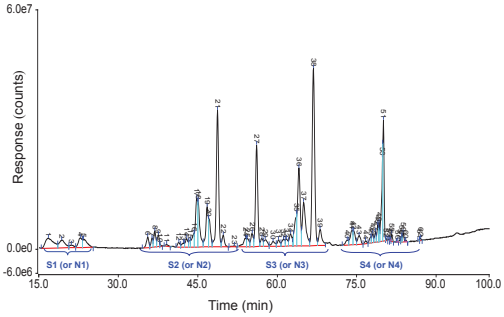
After purification of the labeled oligosaccharides from the unbound labeling reagent, the 2-AB-labeled oligosaccharides can be easily separated using a Thermo Scientific™ Dionex™ CarboPac™ PA1 column. The oligosaccharides were separated using two different gradient elution methods. The first "unmodified" method uses the gradient program shown in Table 1. Figure 3 shows chromatogram obtained for oligosaccharides derived from human α1 acid-glycoprotein using the unmodified elution method.

Figure 3. 2-AB-labeled oligosaccharides from human α1 acid-glycoprotein analyzed using unmodified elution.



The second "modified" elution method involves slight modification of sodium acetate solution used in the gradient. This solution contains 0.05 N sodium hydroxide, which is not present in the unmodified conditions. The goal of this modification is to prevent the microbial contamination that may occur when only sodium acetate is used in he eluent.[4] Table 2 shows the gradient program used for the modified elution. Figures 4 shows a representative chromatogram obtained for oligosaccharides derived from human α1 acid-glycoprotein using modified elution conditions.

Figure 4. AB-labeled oligosaccharides from human α1 acid-glycoprotein analyzed using modified elution.



Figures 5 and 6 show representative chromatograms obtained for oligosaccharides derived from bovine fetuin using unmodified and modified elution conditions respectively.

Figure 5. 2-AB-labeled oligosaccharides from bovine fetuin analyzed using unmodified elution.

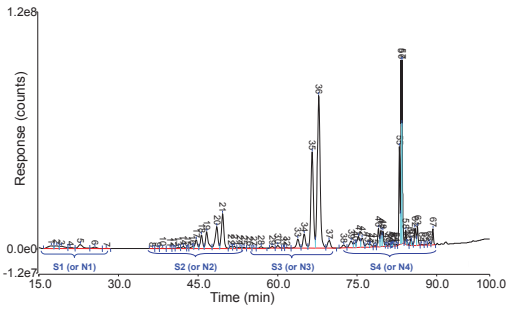
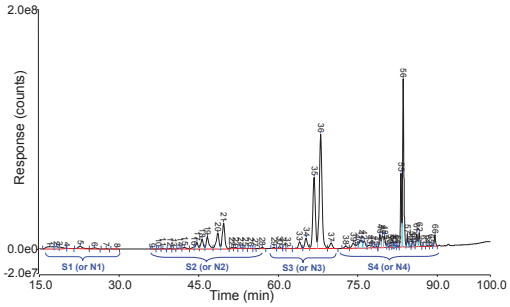


Figure 6. 2-AB-labeled oligosaccharides from bovine fetuin analyzed using modified elution.



The peak groups corresponding to S1, S2, S3, & S4 (mono-sialylated, di-sialylated, tri-sialylated and tetra sialylated, respectively) N-glycans were determined using the retention times of the AB-labeled standards. Then, the average retention time and average relative retention against S4 N-glycans present in the human α1 acid-glycoprotein and fetuin samples were calculated.

Both the average retention time and average relative retention time calculated against A4 oligosaccharide in the samples is used for comparing method performance. Retention time data contained in Tables 3 and 4, for the oligosaccharides derived from both the proteins used in this work, shows only minor difference between the two purification methods as well as the elution methods. This result confirms that both the method variations tested in this work do not significantly affect the elution behavior of the oligosaccharides.

Table 3. Average and relative retention times for *N*-glycans in oligosaccharides released from Human α1 acid-glycoprotein using unmodified as well as modified elution.

N-glycans in Sample	Average Retention Time (min)				Average Relative Retention Time			
	Unmodified Elution		Modified Elution		Unmodified Elution		Modified Elution	
	Glyco-Clean SPE Cartridge	G10 Spin Column	Glyco-Clean SPE Cartridge	G10 Spin Column	Glyco-Clean SPE Cartridge	G10 Spin Column	Glyco-Clean SPE Cartridge	G10 Spin Column
A1	22.17	21.13	20.84	20.70	0.27	0.26	0.26	0.25
A2	42.35	42.25	42.30	42.35	0.52	0.52	0.52	0.52
A3	60.26	60.17	59.91	59.94	0.75	0.75	0.74	0.74
A4	80.02	80.02	80.09	80.09	1.00	1.00	1.00	1.00

Table 4. Average and relative retention times for *N*-glycans in oligosaccharides released from bovine fetuin under unmodified as well as modified elution.

N-glycans in Sample	Average Retention Time (min)				Average Relative Retention Time			
	Unmodified Elution		Modified Elution		Unmodified Elution		Modified Elution	
	Glyco-Clean SPE Cartridge	G10 Spin Column	Glyco-Clean SPE Cartridge	G10 Spin Column	Glyco-Clean SPE Cartridge	G10 Spin Column	Glyco-Clean SPE Cartridge	G10 Spin Column
A1	21.32	21.68	23.06	22.39	0.26	0.26	0.28	0.27
A2	46.29	46.4	46.45	46.6	0.57	0.57	0.57	0.57
A3	63.04	63.11	63.23	63.28	0.77	0.78	0.78	0.77
A4	81.12	80.89	81.0	81.30	1.00	1.00	1.00	1.00

CONCLUSIONS

- The Thermo Scientific Dionex ICS-5000+ system hyphenated with the FLD-3400RS fluorescence detector is a powerful tool for the analysis of 2-AB labeled glycans with Dionex CarboPac stationary phases.
- Using this configuration, separation of 2-AB labeled oligosaccharides was tested with two variations to the method.
- First, separation of labeled oligosaccharides from unbound labeling reagent before chromatographic analysis was achieved using SEC as well as SPE methods and no major effect on retention profiles was observed. Second, elution using two different methods resulted in similar retention times and relative retention time profiles as well.
- This indicates that both the methods used here are equally suitable for analyzing the 2-AB-labeled oligosaccharides.

REFERENCES

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2. Chapter <212> for oligosaccharide analysis proposed by USP specifically deals with profiling of intact cleaved glycan chains in selected recombinant therapeutic glycoproteins.
3. Rohrer, J. S., Basumallick, L., Hurum, D. High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection for Carbohydrate Analysis of Glycoproteins *Biochemistry (Moscow)* **2013** 78, 697-709.
4. Thermo Scientific Technical Note 71: Eluent preparation for high-performance anion-exchange chromatography with pulsed amperometric detection. Sunnyvale, CA.

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