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Glycoprotein Monosaccharide Analysis using HPAE-PAD for Biopharmaceutical Quality Control

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ABSTRACT

Purpose: To develop an accurate method of determining monosaccharides in glycoproteins using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) and manually prepared eluent.

Methods: Glycoprotein hydrolysates were prepared using trifluoroacetic acid and hydrochloric acid. Monosaccharides were detected and quantified using HPAE-PAD.

Results: The proposed method was validated using criteria prescribed in USP <1225>.

INTRODUCTION

Determination of the monosaccharide composition of a glycoprotein pharmaceutical is a typical quality control assay in the pharmaceutical industry. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for glycoprotein carbohydrate analysis. HPAE-PAD separates carbohydrates with specific interactions between their hydroxyl and carboxyl groups based on charge, size, composition, isomerism, and linkages. Therefore, HPAE-PAD is the best method for determining monosaccharides, sialic acids, and other carbohydrates.

HPAE-PAD allows for direct detection without sample derivatization, thereby reducing analyst time, expense, and exposure to hazardous chemicals. It is a selective technique with sensitive detection. Fast separations can be performed without loss of resolution using Thermo Scientific™ Dionex™ CarboPac™ PA20 columns.

The goal of this work is to describe an HPAE-PAD method for monosaccharide composition analysis using manually prepared eluent. Monosaccharide analysis using electrolytically generated eluent has been described before.[1] Here, three different commercially available proteins, IgG, fetuin, and alpha-1-acid glycoprotein (AGP), were individually subjected to two different hydrolysis conditions using 1) HCl, which favors release of amino sugars like galactosamine and glucosamine, and 2) TFA, which favors release of neutral sugars like mannose, glucose, and galactose. Results for method linearity, accuracy, and robustness for monosaccharide quantification are discussed here.

MATERIALS AND METHODS

TFA and HCl Hydrolysis

See Technical Note 40.[1]

Instrument

Thermo Scientific™ Dionex™ ICS-5000+ HPIC™, system with reagent-free ion chromatography (RFIC™) system was used in this work. Figure 1 shows the flowpath of the system, which was configured for carbohydrate detection using electrochemical detection.

In PAD using the four-potential waveform, the disposable working electrode is pulsed through the different potentials at set times, completing two cycles within one second (Figure 2). This waveform is optimized to provide a clean, stable gold layer in preparation for detection of the next eluting peak.

Figure 1.The Dionex ICS 5000+ HPIC system flow diagram configured for ED detection.

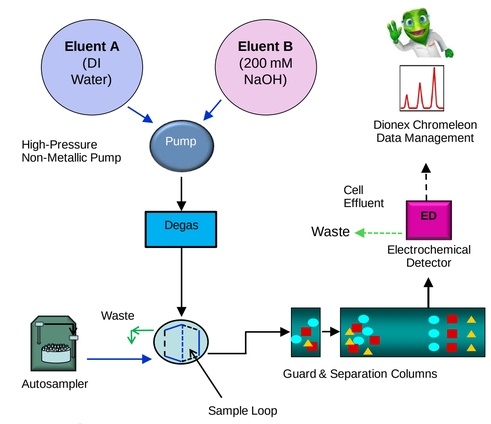
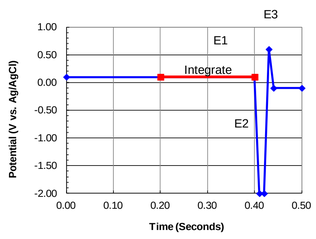


Figure 2. Four-potential carbohydrate waveform.



Time (s)	Potential (V)	Integration
0.00	+0.10	
0.20	+0.10	Start
0.40	+0.10	End
0.41	-2.0	
0.42	-2.0	
0.43	0.60	
0.44	-0.10	
0.50	-0.10	

1. Detection potential (E1)
2. Duration time of E1 (t1)
3. Integration period (Integrate)
4. Reductive cleaning potential (E2)
5. Duration time of E2 (t2)
6. Oxidative cleaning potential (E3
7. Duration time of E3 (t3)
8. Pre-detection (oxide reduction) potential (E4)
9. Duration time of E4 (t4)

Data Analysis

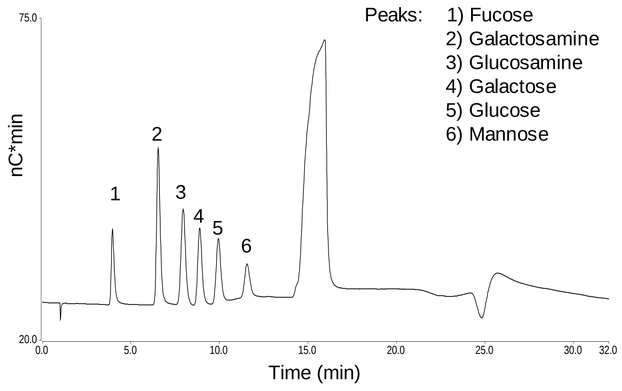
Thermo Scientific™ Chromeleon™ CDS software, version 7.2 SR4.

RESULTS

Separation

Separation of monosaccharides was achieved using a Dionex CarboPac PA20 column (3 × 150 mm) with a Thermo Scientific™ Dionex™ AminoTrap™ guard column using isocratic elution conditions, followed by a step change to higher eluent concentration, which was used to remove contaminant species, including carbonate, still bound to the column. Figure 3 shows a typical separation of a 10 µL injection of the Thermo Scientific™ Dionex™ MonoStandard, containing fucose, galactosamine, glucosamine, galactose, glucose, and mannose, each at 10 µM concentration (100 pmol each). The peaks are baseline resolved and elute within a window of 13 min. The total run time is 32 min to allow for washing and re-equilibration after the column regeneration step.

Figure 3. Separation of a 10 µM Dionex MonoStandard injection containing 10 µM each of the six monosaccharides.

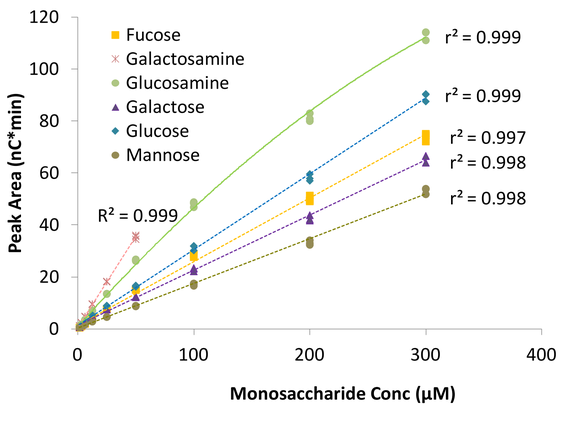


Columns: Dionex CarboPac PA20 Analytical, 3 × 150 mm
Dionex AminoTrap Guard 3 × 30 mm
Flow Rate: 0.5 mL/min
Inj. Volume: 10 L
Column Temp: 30 ° C
Tray Temp: 4 ° C
Eluent : 10 mM NaOH from 0 to 12 min; 200 mM NaOH for 12 to 22 min; 10 mM NaOH from 22 to 32 min.

Linearity and Precision

The linearity of monosaccharide determination was studied by generating peak area response curves for all six monosaccharides using a standard mix containing 1.56 to 300 µM of each of the six monosaccharides, except galactosamine for which the linearity range was from 1.56 to 50 µM. The results included in Figure 4 show that the coefficients of determination ranged from 0.997 to 0.999 for all six monosaccharides. Linear curve fits were used for all monosaccharides except glucosamine, for which a quadratic curve fit was used.

Figure 4. Monosaccharide peak area calibration plots (n=3).



Method precision was determined in two ways. First, method repeatability was determined at three concentration levels of the six Dionex MonoStandard mix with three replicates of each sample to give nine total injections. Excellent peak area as well as retention time precision was obtained for all three concentration levels tested with all RSD values below 2% (Table 1).

Table 1. Method precision determined at three concentrations (n=3).

Standard Conc	Retention Time and Peak Area RSDs											
	Fucose		Galactosamine		Glucosamine		Galactose		Glucose		Mannose	
	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area
3.12 µM	0.12	1.14	0.07	1.84	0.01	1.57	0.01	1.78	0.05	1.89	0.07	0.73
12.5 µM	0.13	1.12	0.00	0.91	0.06	0.92	0.01	1.06	0.05	1.08	0.05	0.95
100 µM	0.00	1.67	0.00	1.46	0.06	1.42	0.01	1.22	0.00	1.33	0.01	1.07

The intermediate precision was determined by assaying eight replicate injections of the 10 µM Dionex Mono-Standard each day for three consecutive days. The results of this experiment contained in Table 2 show excellent retention time as well as peak area precision

Detection Limits

The monosaccharide detection limits for this HPAE-PAD assay under the conditions described was set at monosaccharide concentrations that resulted in a signal-to-noise ratio of 10:1. A series of monosaccharide standards were prepared and analyzed. The signal-to-noise ratios were calculated using the peak height for each monosaccharide; the noise level was calculated from a stable portion of the baseline where no peak elutes. Table 3 contains limits of detection for all six monosaccharides determined in this study.

Table 2. Method intermediate precision (n=8).

Days	Retention Time and Peak Area RSDs											
	Fuc		GalN		GlcN		Gal		Glu		Man	
	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area
1	0.2	2.0	0.4	2.2	0.5	2.1	0.3	1.9	0.5	2.6	0.6	1.9
2	0.0	3.4	0.0	3.4	0.2	3.4	0.1	2.9	0.0	3.0	0.1	3.4
3	0.1	3.0	0.1	2.6	0.1	2.6	0.1	1.8	0.1	2.6	0.1	2.1

Table 3. Method sensitivity determination (n=3).

Monosaccharide	Detection Limit (µM)	Amount Injected (pmoles)	S/N Ratio
Fucose	0.25	2.5	13.4
Galactosamine	0.1	1.0	10.9
Glucosamine	0.2	2.0	9.7
Galactose	0.25	2.5	13.7
Glucose	0.25	2.5	11.7
Mannose	0.2	2.0	9.5

Accuracy

Accuracy of the assay was determined by spiking known amount of monosaccharides into each of the dried and reconstituted acid hydrolysates prepared for the three glycoproteins used in the study. Figure 5 shows representative chromatograms for HCl and TFA hydrolysates for AGP.

For each monosaccharide, 20% to 150% spike levels based on calculated endogenous monosaccharide concentration were used. The monosaccharides present below the lowest calibration standard were not quantified and hence were not spiked. The results in Figure 6 show excellent recoveries of the spiked monosaccharides with all the recoveries between 80% and 120%.

Figure 5. AGP oligosaccharide TFA and HCl hydrolysate analysis.

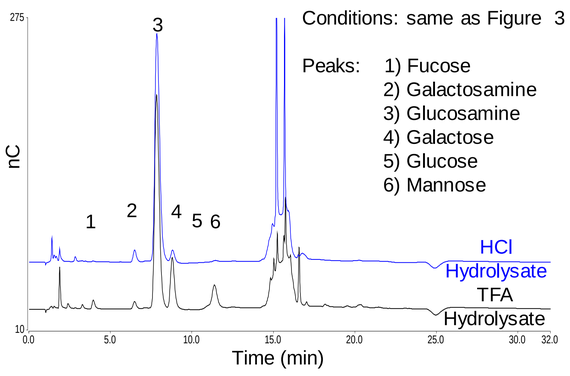
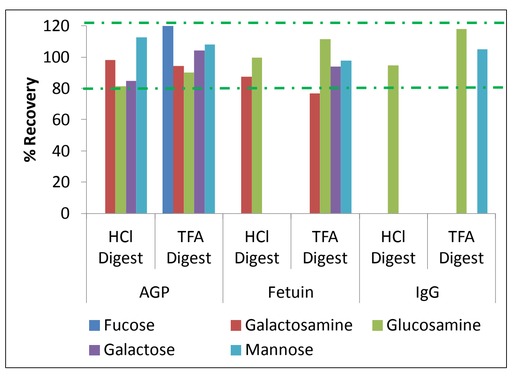


Figure 6. Monosaccharide spike recovery (n=3).



Robustness

Assay robustness was determined on three columns, two new columns from the same lot and a six-month-old column from a different lot. The robustness was studied by introducing ±10% variation in common chromatographic parameters. The parameters varied in this study were: initial eluent concentration, final eluent concentration, column temperature, and flow rate. Method performance under these conditions was evaluated by injecting 10 µM Dionex MonoStandard and calculating percent difference in three key chromatographic parameters: retention time, peak asymmetry, and resolution.

Table 4 contains representative data from column 1, which shows minimal disruption in chromatographic parameters. Results from columns 2 and 3 showed similar trends (not shown).

Table 4. Results of robustness study performed on column 1 (n=3).

Condition	Percent Difference (%)										
	Retention Time						Resolution				
	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc
-10% Initial Eluent	1.5	2.7	3.3	2.5	3.0	4.0	2.3	2.4	-6.5	4.6	7.1
+10% Initial Eluent	-0.7	-1.7	-2.2	-1.5	-1.9	-2.6	-2.0	-2.4	6.8	-3.3	-4.3
-10% Column Temp.	2.2	5.6	5.9	4.1	4.9	5.0	3.9	-2.1	-18.5	5.9	-0.5
+10% Column Temp.	-2.7	-6.2	-6.5	-4.5	-5.3	-5.7	-6.5	0.0	21.7	-6.5	-0.3
-10% Flow Rate	12.0	12.4	12.7	12.4	12.6	13.1	2.4	3.4	0.1	4.0	7.2
+10% Flow Rate	-8.4	-8.3	-8.0	-8.3	-8.1	-7.8	-2.4	-1.3	-5.5	-0.4	0.8
-10% Final Eluent	0.2	-0.0	0	-0.1	0	0	-1.4	-0.2	-0.9	0.1	0.6
+10% Final Eluent	0.3	0.2	0.32	0.1	0.2	0.3	-1.2	0.1	-2.3	0.8	1.3

CONCLUSIONS

- The high resolution separation of all six sugars was achieved in 13 min. Total run time is 32 min.
- The method accuracy was determined by measuring the monosaccharide content of the example glycoproteins.
- The method was validated as per analytical performance characteristics outlined in USP General Chapter <1225>.

REFERENCES

1. Thermo Scientific Technical Note 40 (TN40). Glycoprotein monosaccharide analysis using HPAE-PAD with eluent generation (HPAE-PAD). [Online] https://www.thermofisher.com/content/dam/tfs/ATG/CMD/CMD%20Documents/Application%20&%20Technical%20Notes/Chromatography/Ion%20Chromatography/IC%20and%20RFIC%20Columns/5052-TN40-IC-Glycoprotein-Monosaccharide-23May2012-LPN1632-01.pdf (Accessed January 9, 2017).

TRADEMARKS/LICENSING

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