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Analysis of 2-Aminobenzamide (AB) Labeled Glycans Using HPLC with Fluorescence Detection

INTRODUCTION

The study of a protein's glycosylation can reveal highly valuable diagnostic information. For instance, several studies report the identification of protein glycans as biomarkers of life-threatening diseases such as cancer.¹⁻³ In addition, glycomics studies may have the potential to reveal predisposition to some diseases.⁴ Glycoprotein analysis is of paramount importance for the biopharmaceutical industry. Glycosylation has a profound impact on many physiochemical properties of proteins that can affect both efficacy and safety. According to International Conference on Harmonization (ICH) guidelines, structure, content, and glycosylation sites should be investigated as thoroughly as possible.⁵

Within protein glycosylation studies, the most common approach to qualitative and quantitative characterization of glycans is the analysis of glycans enzymatically or chemically released from the protein. This approach leads to mixtures of oligosaccharides that are often quite complex and require high-efficiency separation techniques prior to characterization, usually accomplished by mass spectrometry (MS). Ultraviolet (UV) detection cannot be used at this stage because glycans lack chromophores; this poses a problem when the separation technique is not directly hyphenated to MS. The problem can be overcome by using high-performance anion-exchange with pulsed amperometry detection (HPAE-PAD), which combines a highly efficient separation with an effective detection strategy, sensitive to the low picomole (pmol) level.^{6,7}

A second approach is to label the glycans with a fluorescent molecule, followed by high-performance liquid chromatography with fluorescence detection (HPLC-FD) analysis. Sample preparation is more time consuming with this approach, due to the tagging procedure and sample cleanup. However, HPLC-FD is still preferred to HPAE-PAD when sensitivity is critical and when fractions obtained from the separation require analysis by MS. Fractions collected from HPAE-PAD separation are rich in salts and require desalting before MS analysis.

Among the fluorescent molecules that can be used for tagging carbohydrates, 2-aminobenzamide (2-AB) is the most commonly used when HPLC is the selected separation technique. The tagging protocol with 2-AB is fairly simple, causes negligible desialylation, and is quantitative.⁸ Moreover, glycans and 2-AB are strongly bonded, thus the tagged molecules have excellent stability, provided they are not dissolved in strong acidic solutions and/or stored at high temperatures. Because 2-AB-glycans are hydrophilic compounds, the HPLC separation technique most frequently chosen is hydrophilic interaction liquid chromatography (HILIC). Use of reversed-phase (RP) separation has been reported,⁹ but retention is generally poor in comparison to HILIC.

The most successful HILIC phase for this purpose is amide-modified silica, which delivers good retention and selectivity for both neutral and (multiple) charged glycans in one separation. This type of stationary phase is currently preferred for the separation of 2-AB glycans. A database that allows 2-AB-glycans structure assignment based on the retention time obtained with coated amide columns has been created;¹⁰ consequently, the impact of this stationary phase on glycan analysis has greatly increased.

This work shows separations of complex mixtures of 2-AB labeled glycans using a Thermo Scientific Dionex UltiMate™ 3000 RSLC system implemented with an FLD-3400RS fluorescence detector and amide column operated in HILIC mode. Retention time, peak area, limits of detection (LOD), and low- and high-level linearity for these labeled glycans are provided.

EQUIPMENT

Dionex UltiMate 3000 RSLC system including:

- SRD-3400 Solvent Rack (P/N 5035.9245)
- HPG-3400RS Pump upgraded to 1034 bar with 350 μ L mixer (P/N 5035.9250)
- WPS-3000TRS Autosampler (P/N 5840-0020)
- Sample loop, 25 μ L (P/N 6820.2415)
- Needle seat capillary, 1.35 μ L (P/N 6820.2407)
- TCC-3000RS Column Compartment (P/N 5730.0000)
- Thermo Scientific Dionex nanoViper™ capillary 550 mm, 75 μ m i.d. (P/N 6041.5760)
- FLD-3400RS Fluorescence Detector with dual photo multiplier tube (PMT) (P/N 5078.0025)
- Flow Cell for FLD-3400RS, 8 μ L (P/N 6078.4230)
- Flow Cell for FLD-3400RS, 2 μ L (P/N 6078.4330)
- Thermo Scientific Dionex Chromeleon™ Chromatography Workstation, 6.8 or higher
- Polypropylene vials with glass insert, 250 μ l, 25 pcs (P/N 163974)
- Polypropylene caps, 100 pcs (P/N 160135)
- TSK-Gel Amide-80 column, 3 μ m, 2.0 \times 150 mm (Tosoh P/N 21865)

REAGENTS AND MATERIALS

- Deionized (DI) water, 18 M Ω resistivity or higher
- Acetonitrile (Biosolve BV P/N 012035)
- Ammonium formate (Sigma Aldrich P/N 17843)
- Formic acid (Sigma-Aldrich P/N 06440)
- 2-AB tagging kit (Ludger P/N LT-KABA2)
- 2-AB glycans cleanup cartridges (Ludger P/N LC-D1-30-A6)

STANDARDS

- Fetuin *N+O* glycan library (Ludger P/N CLIBNO-FETUIN-01)
- 2-AB labeled *N*-glycan library from bovine fetuin (Prozyme P/N GKSB-002)
- 2-AB labeled human IgG *N*-glycan library (Prozyme P/N GKSB-005)
- 2-AB labeled di-sialylated, galactosylated biantennary glycan (Prozyme P/N GKSB-312)
- 2-AB labeled mono-sialylated, galactosylated biantennary glycan (Prozyme P/N GKSB-312)
- 2-AB labeled asialo, galactosylated biantennary glycan (Prozyme P/N GKSB-304)

ABBREVIATIONS

- 2-AB-NA2: 2-AB labeled asialo, galactosylated biantennary glycan
- 2-AB-A1: 2-AB labeled mono-sialylated, galactosylated biantennary glycan
- 2-AB-A2: 2-AB labeled di-sialylated, galactosylated biantennary glycan

PREPARATION OF SAMPLES AND STANDARDS

Tag the bovine fetuin *N,O*-glycan library using the tagging kit provided by Ludger according to the vendor instructions. Purify the sample with the appropriate cleanup cartridges according to the vendor instructions. Dissolve dry samples of standard 2-AB labeled glycans and IgG 2-AB-*N*-glycans with the appropriate amount of 8/2 acetonitrile/ammonium formate 0.1 M, pH 4.5.

Store all samples at -20 °C.

Preparation of Ammonium Formate, 0.1 M, pH 4.5

Dissolve 6.03 g ammonium formate in 1 L of DI water. Adjust the pH to 4.5 by carefully adding formic acid.

SETTING THE FLD-3400RS DETECTOR

The excitation and emission wavelengths used throughout the experiments are 320 nm and 420 nm, which correspond to the maximum of the excitation and emission spectra of 2-AB. For sensitive detection of trace amounts of labeled glycans, set the sensitivity of the PMT to 8, which is the instrument maximum. Set the lamp mode to high energy.

The emission signal stray light can be suppressed by setting the appropriate cut-off filter wheel. Several options are available in the FLD-3400: 280, 370, 435, and 530 nm cut-off filters.

RESULTS AND DISCUSSION

Separation of Mixtures of 2-AB Labeled Glycans

The glycans released from bovine fetuin are heterogeneous in terms of charge because their sialic acid content can vary between 0 to 5 units. The human IgG sample is less heterogeneous, consisting primarily of neutral and mono-sialylated glycans (Figure 1). This work shows that these diverse glycan samples can be successfully separated using the Dionex UltiMate 3000 RSLC system.

More than 20 peaks were detected in the sample of fetuin 2-AB-(*N,O*)-fetuin glycans. All peaks eluted within 30 min (Figure 2). The IgG 2-AB-glycans were separated with a shorter run: 10 peaks were resolved in <10 min, as shown in Figure 3.

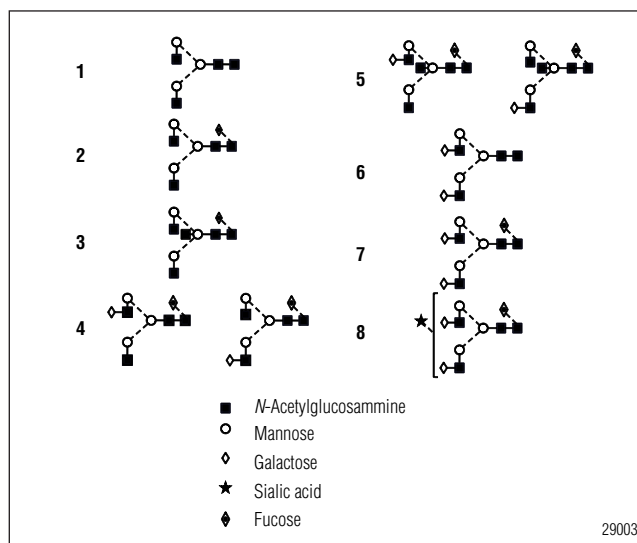


Figure 1. Glycan structures corresponding to the peaks numbered in Figure 3. Assignments were made based on published papers.

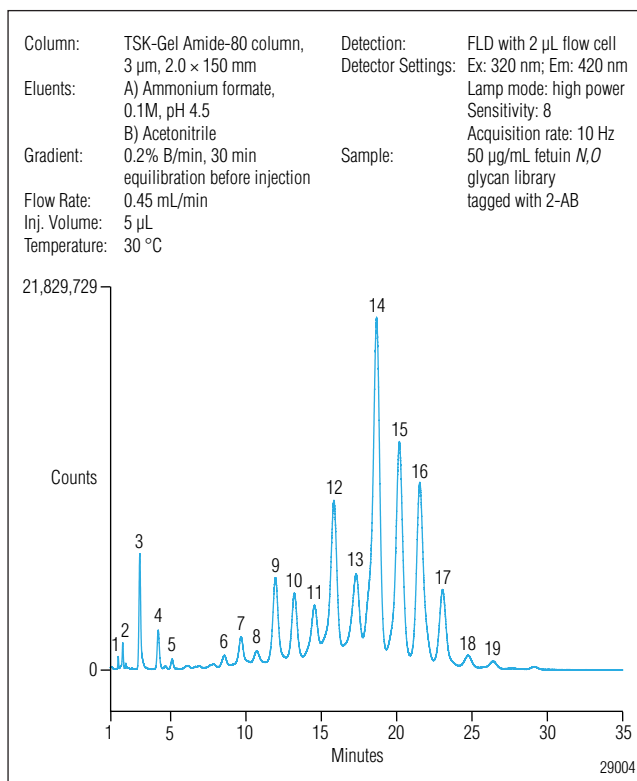


Figure 2. Separation of bovine fetuin 2-AB-N,O-glycans.

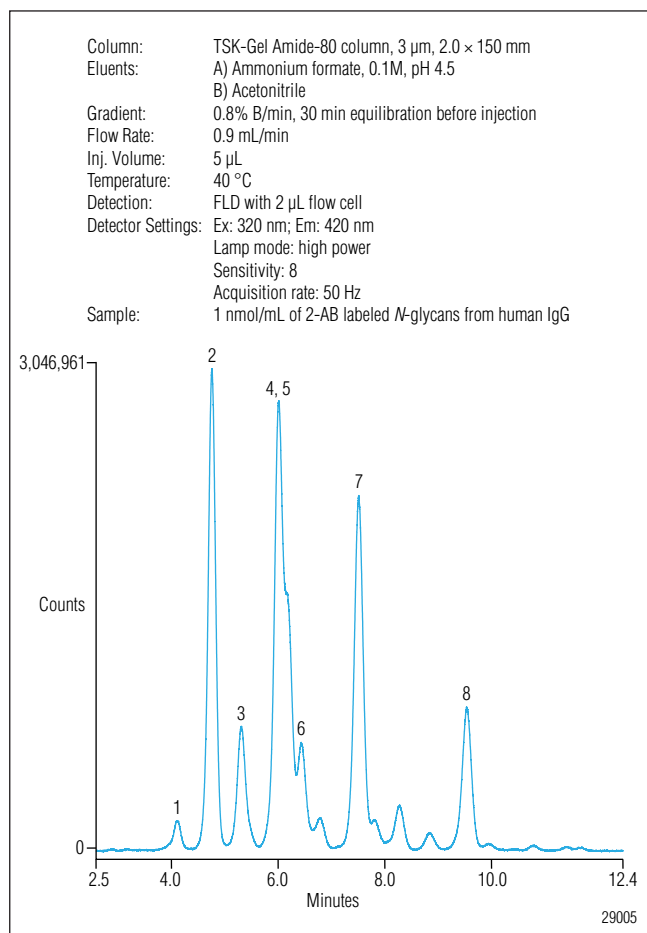


Figure 3. Separation of 5 pmol human IgG 2-AB-glycans.

Retention Time and Area Precisions

Retention time precision is essential in labeled glycan analysis. This applies particularly to experiments where the relative retention times are expressed based on the retention time of glucose oligomers, with the purpose of structure assignment based on GlycoBase database.¹⁰ A method with repeatable retention times allows the injection of the unknown sample and the glucose ladder standard in separate runs, avoiding the practice of spiking the sample with the glucose standard, hence eliminating the risk of co-elution between glucose oligomers and analyte peaks.

Retention time precision was measured for repeated injections of the fetuin 2-AB-*N,O*-glycans. The peaks numbered in Figure 2 were evaluated. For all peaks, the RSD was below the value 0.3% commonly reported¹¹ (Figure 4). In addition, retention time precision was evaluated for injections of single standard (2-AB-NA2); an RSD of 0.09% was measured in this case. Retention time precision data are shown in Table 1.

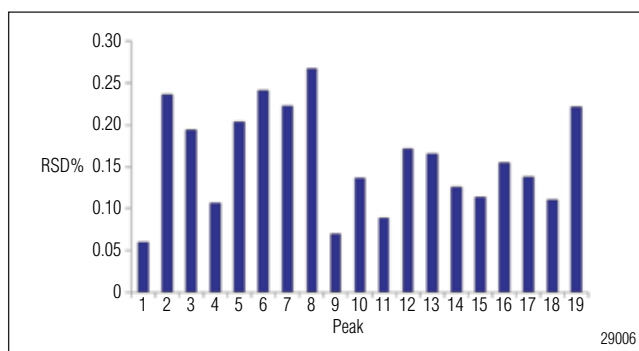


Figure 4. Retention time precision for five consecutive injections of the peaks numbered in Figure 2.

Table 1. Retention Time Precision (n=5)			
	Fetuin 2-AB- <i>N,O</i> -glycans (Average 19 Peaks)	Fetuin 2-AB- <i>N,O</i> - glycans (Peak #1)	2-AB-NA2
Retention Time Precision (RSD%)	0.16	0.06	0.09

Same chromatographic conditions as in Figure 2.

Area precision was evaluated for five consecutive injections of 2-AB-NA2, and the measured RSD was 0.56%.

Linearity

Linearity (Table 2) was evaluated for two ranges: 0.05–1.25 pmol and 0.5–12.5 pmol, with 2-AB-NA2 and 2-AB-A1, respectively. Linearity was excellent in both ranges and interpolation resulted in r^2 well above 0.99.

Table 2. Linearity Obtained at Two Levels with Different Samples (n=5)		
Sample	Range (pmol)	r^2
2-AB-NA2	0.05–1.25	0.9983
2-AB-A1	0.50–12.5	0.9999

Same chromatographic conditions as in Figure 2.

LOD and LOQ

Limits of detection (LOD) and limits of quantitation (LOQ) were measured by a calibration line of peak height as a function of the quantity of standard injected. The calibration line was drawn in the range between 20–200 fmol. The LOD and LOQ were calculated according to the following definitions:

$$LOD = 3.3 \times \frac{\sigma}{S} ; LOQ = 10 \times \frac{\sigma}{S}$$

where σ is standard deviation of the y-axis intercept and S is the slope of the calibration line. Detection and quantification limits were calculated with the 2 μ L and 8 μ L flow cell. In both cases, the calibration linearity was excellent ($r^2 > 0.99$). Limits of detection of approximately 3 fmole and 19 fmole were measured for the 8 μ L and 2 μ L flow cells, respectively. The LOQ was 10 fmole and 58 fmole for the 8 μ L and 2 μ L flow cells, respectively (Table 3).

CONCLUSION

The Dionex UltiMate 3000 RSLC system hyphenated with the FLD-3400RS fluorescence detector is a powerful tool for the analysis of 2-AB labeled glycans with amide HILIC stationary phases. Extremely low detection limits (< 5 fmol) were observed with an analytical 8 μ L flow cell. The implementation of a 2 μ L flow cell yielded highly sensitive determinations and simultaneously improved the efficiency of the method by decreasing the extracolumn band dispersion; half-height peak width

values of Table 3 and an overlay of peaks in Figure 5 clearly show this effect. With the column format used here (2.1 mm i.d. and 3 μ m particle size), the 8 μ L flow cell is recommended for methods with low amounts of sample and well-resolved peaks, whereas the 2 μ L flow cell is recommended when higher resolving power is required.

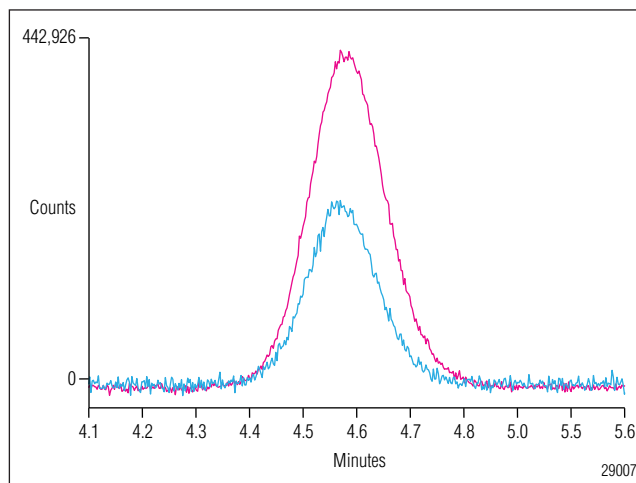


Figure 5. Overlay of emission signals of 2-AB-NA2 (200 fmol) obtained with the 2 μ L (small peak) and 8 μ L (large peak) flow cell. Other conditions are the same as in Figure 2

Linearity of the method was excellent for detection of samples in the range spanning from low fmol to low pmol. The linearity range may extend to higher concentrations; however, they were not investigated for this study.

The precision of the method is satisfactory from both a quantitative and qualitative standpoint. The Dionex UltiMate 3000 RSLC system can be effectively used for separation of labeled glycans and peak recognition by GlycoBase.

Table 3. Results of the LOD and LOQ with Calibration Curve

Flow Cell	Calibration Range (fmol)	Coefficient of Determination (r^2)	LOD (fmol)	LOQ (fmol)	Half-Height Peak Width (sec)
2 μ L	20–200	0.9949	19.1	58.0	12.6
8 μ L	20–200	0.9998	3.3	10.0	13.7

Peak width at half height was evaluated for the peak obtained with the 200 fmol sample. Same chromatographic conditions as in Figure 2.

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