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Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 μm sorbent

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ABSTRACT

Separation by hydrophilic interaction chromatography (HILIC) with fluorescence detection utilizing a sub-2 μm glycan column for the separation of 2-aminobenzamide (2-AB) labeled N-linked glycans is described. The HILIC column packed with a 1.7 μm amide sorbent improves the peak capacity compared to a 3.0 μm HILIC column by a similar degree as observed in reversed-phase ultra-performance liquid chromatography (RP-UPLC). The results indicated that the optimal peak capacity was achieved at flow rate 0.2–0.5 mL/min. HILIC method transfer guidelines were shown to further enhance the resolution of glycans by changing initial gradient conditions, flow rate, column temperature, and different column lengths. Additionally, excellent resolution can be achieved in the separation of 2-AB labeled glycans released from fetuin, RNase B, and human IgG with a rapid analysis time.

1. Introduction

Glycoprotein characterization and glycosylation profiling are important tasks in the development and production of biopharmaceutical proteins. Useful information about protein glycoforms can be obtained by top-down analysis of intact glycoproteins [1], analysis of glycopeptides [2], or after glycan release from proteins [3,4]. Among the methods used for glycan quantitation are high-performance anion-exchange chromatography (HPAEC) with fluorescent [5] or pulsed amperometric detection (PAD) [3,6], reversed-phase liquid chromatography (RP-LC) [7], capillary electrophoresis (CE) [8], and hydrophilic interaction chromatography (HILIC) [3].

The acronym HILIC was proposed by Alpert [9] and refers to liquid chromatography utilizing a polar stationary phase (e.g. amide, diol, or cyano phases) and mobile phases using a high percentage of acetonitrile. The strongly retained polar analytes are eluted with an aqueous gradient. The same types of stationary phases have been traditionally used for normal phase (NP) chromatography, and some authors use NP terminology to describe the separation of glycans. In this publication we refer to the method as HILIC.

The HILIC method for analysis of glycans has gained considerable attention in the last decade, primarily due to the high detection sensitivity provided by fluorescent detectors [3,10], good reproducibility [11], and the ability to separate positional isomers [11–13]. In addition, the available database tools such as GlycoBase and AutoGU permit tentative assignments of glycans and their structures from glucose unit values (GU). The GU values for labeled glycans are obtained by normalizing their LC retention times against a dextran ladder standard [7,10,11].

Some laboratories utilize a combination of LC methods to create two-dimensional (2D), or three-dimensional (3D) maps to identify highly complex glycan samples. The most common approaches combine HILIC and RP-LC separation modes [7], or HILIC and HPAEC methods [14,15], where anion-exchange chromatography is utilized to fractionate glycans according to their charge or number of sialic acid residues in the glycan structure. Combinations of all three LC separation modes with normalized retention time GU plots have been reported [7].

Liquid chromatography combined with mass spectrometry (MS) has also been used for glycan characterization. Several technical drawbacks limit the application of MS technology for glycan analysis. Electrospray ionization (ESI) of carbohydrates is difficult due to the low ionization efficiency; hence the minor components in the sample are often undetectable. In addition, the mobile phases used with HPAEC or HILIC methods are poorly compatible with ESI [4,16].

LC–MS has been performed in RP mode with ESI-MS compatible mobile phases. Highly retentive graphitized carbon columns are useful for analysis of unlabeled glycans [17]. To improve the limits of MS detection, the analysis is often performed in micropore, capillary or nano-LC format [18,19].

Recent progress in the development of ultra-performance liquid chromatography (UPLC) technology allows for an enhanced resolu-

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tion of complex samples [20]. Development of columns packed with sub-2 μm particle sorbents has enabled significant improvements in separation power, often defined as peak capacity for peptides, oligonucleotides, and other analytes [21,22]. The introduction of UPLC columns for HILIC mode [23] offers prospects for similar improvements in the separation of polar molecules such as glycans.

In this paper we investigate the peak capacity of HILIC columns packed with a sub-2 μm amide sorbent using various flow rates, initial gradient conditions, and column temperatures. While the peak capacity theory in the RP-LC is well understood, limited data are available for the HILIC mode. Using the ranges of conditions providing the best peak capacity we further modified the gradient conditions with the aim of altering separation selectivity and achieving optimal resolution of selected labeled glycans. Obtained conditions were applied to separate mixtures of glycans; UPLC and HPLC columns were compared. Finally, we provide method transfer guidelines for UPLC HILIC columns of different lengths.

2. Experimental

2.1. Materials and reagents

Ammonium formate, ribonuclease B (RNase B), peptide N-glycosidase (PNGase F) and GlycoProfile 2-AB-labeling Kit were purchased from Sigma (St. Louis, MO, USA). Formic acid was purchased from EMD chemicals (Gibbstown, NJ, USA). HPLC grade acetonitrile (MeCN) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). A Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare deionized water (18 MΩcm) for HPLC grade mobile phases. Dextran standard 5000 was obtained from Fluka (Steinheim, Denmark). MassPREP Glycoanalysis Kit containing RapiGest SF, 96-well microElution HILIC solid phase extraction (SPE) plate, and MassPREP MALDI matrix 2,5-dihydroxybenzoic acid (DHB), was obtained from Waters Corp. (Milford, MA, USA). 2-AB labeled human IgG N-linked glycans (part #GKSB-005, lot #DP08K1901) and 2-AB labeled Bovine fetuin N-linked glycans (part #GKSB-002, lot #DP06A0406) purchased from ProZyme (San Leandro, CA, USA).

2.2. Standard and sample preparation

RNase B was denatured in 0.1% (w/v) RapiGest SF solution in 50 mM ammonium bicarbonate and reduced with 10 mM dithiothretol and incubated at 65 °C for 30 min. Deglycosylation of N-linked glycans was performed by addition of PNGase F at 1:50 (w/w, enzyme:protein) and incubated at 37 °C overnight. Released glycans were extracted using microElution HILIC SPE 96-well plate using protocol as described earlier [24]. The HILIC plate retains released glycans while removing the remaining protein and denaturant. The extracted glycans were subsequently labeled with 2-aminobenzamide (2-AB) using Sigma GlycoProfile 2-AB labeling kit. Following the labeling reaction, the microElution HILIC plate was used again to extract 2-AB labeled glycans and remove the excess of labeling reagents. Verification of purified free glycans and labeled glycans was confirmed by Waters MALDI MX Tof MS. 2-AB labeled dextran ladder standard containing glucose unit (GU) oligomers, was prepared in a similar fashion as 2-AB glycan samples. The labeled dextran ladder standard was dissolved in the condition matching the initial organic concentration of HILIC gradient, which help prevent the peak broadening of early eluents.

Fetuin 2-AB labeled glycans shown in Supplemental Fig. 3, was prepared by following the protocols of N-linked glycan release, 2-AB labeling, and exoglycodase array digestions as described earlier [25].

2.3. Instrumentation and columns

HILIC separation of 2-AB labeled glycans was carried out using an ACQUITY UPLC system with an ACQUITY UPLC fluorescence (FR) detector (Waters, Milford, MA, USA). The HILIC columns were ACQUITY UPLC BEH Glycan columns packed in 2.1 mm × 50 mm, 100 mm, and 150 mm configurations packed with 1.7 μm amide sorbent (Waters). The weak and strong wash solvents in the ACQUITY UPLC Sample Manager were used to eliminate carry-over. The composition of weak wash solvent was same as the initial organic concentration of HILIC gradient and the strong wash solvent was prepared in 20% MeCN. TSKgel Amide-80 column packed with 3.0 μm particles in 2.0 × 150 mm (TOSOH Bioscience, PA, USA) was run in Alliance 2695 system with Alliance fluorescence 2475 detector (Waters, Milford, MA, USA). The fluorescence detector excitation and emission wavelengths were set at 330 and 420 nm, respectively. Mass spectrometry analysis was carried out on a Waters Synapt QTof MS equipped with an ESI source operated in the positive ion mode. The MS was set at capillary 3.7 kV, sample cone voltage 36 V, source temperature 120 °C, desolvation temperature 350 °C, and desolvation gas flow 800 L/h. The UPLC-FL system was controlled by Waters Empower 2, the LC–MS analysis was controlled by MassLynx 4.1 software.

2.4. Hydrophilic interaction chromatography

Several HILIC gradient conditions were developed for the 2-AB labeled glucose ladder standard, and 2-AB labeled glycans released from fetuin, RNase B and IgG. All separations were performed using 100 mM ammonium formate, pH 4.5 as solvent A and 100% MeCN as solvent B. For 2-AB glucose standard, the gradient started from 70 to 40% MeCN for 15 min (gradient #1) followed by column wash at 80% MeCN for 4 min and finally the column was re-equilibrated at 70% MeCN prior to next injection. The gradient was performed at 12 different flow rates from 0.1 to 1.0 mL/min using two columns packed with 1.7 and 3.0 μm sorbents (from Waters) in 2.1 mm × 50 mm column. Prototype 3.0 μm particles were prepared as a small research batch. We evaluated the impact of column temperature on separation using 40 and 60 °C. Selected gradient conditions were applied for analysis of Fetuin, RNase B and IgG glycans (see figure captions). Mobile phases with reduced concentration of ammonium formate buffer were utilized in order to improve limits of LC–MS detection. The solvent A concentration was either 0.5% formic acid, or variable ammonium formate concentration (1–100 mM) at pH 4.5.

3. Results and discussion

3.1. Experimental evaluation of column peak capacity

The HILIC separation of a 2-AB labeled dextran ladder is shown in Fig. 1. The 2-AB labeled dextran ladder standard is often used for glycan retention time normalization, converting retention times to glucose units. The standard used in this work contains a mixture of peaks containing n-number of glucose monomers ($2 < n < 18$) in linear chain, and represents a suitable sample for following peak capacity experiments.

Linear glucose oligomer peaks elute in the order of their size (molecular weight, MW), shorter oligomers first as illustrated in Fig. 1. Peaks 8 and 11 denote analytes with 8 and 11 glucose monomers in GU, respectively. In order to investigate the impact of the flow rate on peak capacity, we used a gradient from 70 to 40% MeCN in 15 min at various flow rates using a 2.1 mm × 50 mm, 1.7 μm column.

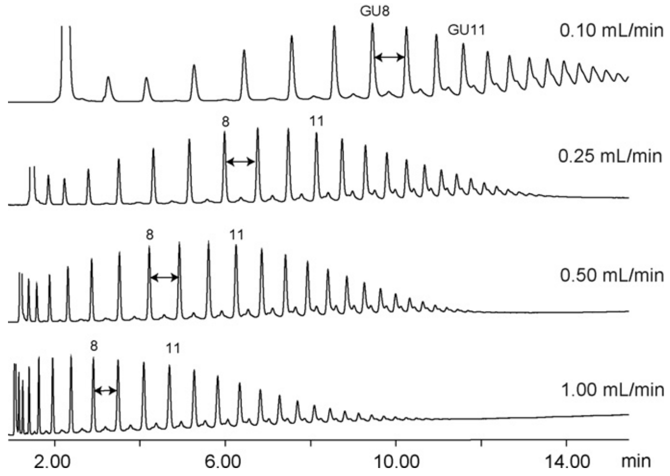

Fig. 1. 2-AB labeled Dextran ladder standard separations at 0.10, 0.25, 0.50, and 1.00 mL/min. Conditions: 2.1 mm × 50 mm, 1.7 μm, column temperature at 60 °C, gradient starting from 70 to 40% MeCN in 15 min. The GU 8–9 and GU 11–12 peaks were used for calculation of peak capacity values using Eq. (1).

The peak capacity P_c was calculated from retention times and widths of selected peaks as specified in Eq. (1):

$$P_c = 1 + \frac{t_2 - t_1}{(w_1 + w_2)/2} \qquad (1)$$

The denotation t_1 and t_2 represent the retention times of selected peaks, e.g. peaks 8 and 9 in Fig. 1. Similarly, w_1 and w_2 represent the peak widths measured at 2.355σ (50% peak height). We chose to measure the peak widths at 2.355σ peak height rather then 4σ (13.4% peak height) because of the minor isomers eluting on the shoulder of the main peaks.

Comparison of chromatograms at various flow rates for the 2-AB labeled dextran ladder standard is shown in Fig. 1. It is apparent that the peak widths decrease, however, the reduction of the overall retention window (separation space) at higher flow rates should also be taken into a consideration.

Peak capacity calculated using Eq. (1) is shown in Fig. 2. The curves exhibit a shallow maximum at ~0.4 mL/min, which represents an optimal flow rate for selected analytes. The curves for glycans in HILIC closely resemble the ones obtained in RP-LC for peptides of similar molecular weight [21].

The gradient theory suggests that maximum peak capacity in gradient elution lies above the optimal mobile phase linear velocity dictated by Van Deemter curves [21,22]. This is due to the contribution of the gradient slope which becomes shallower at faster flow rates and partially compensates for the loss of separation efficiency, *N*, at flow rates above the Van Deemter optimum. We have estimated the optimal flow rate for peak 8 using pseudo Van Deemter curves as described by Gilar and Neue [21]. The optimal column efficiency for 2.1 mm × 50 mm, 1.7 μm HILIC column was found to be below 0.1 mL/min (data not shown).

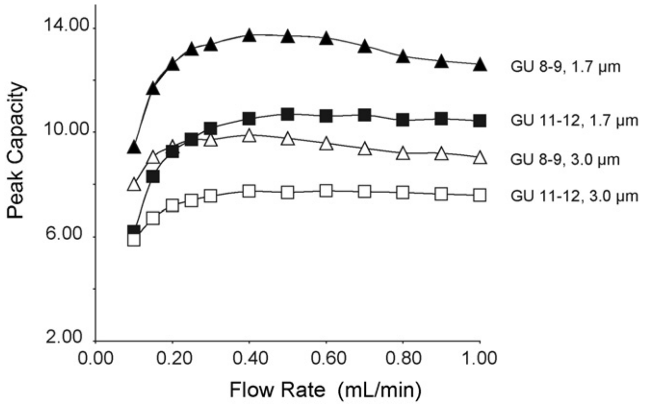

Fig. 2. Evaluation of peak capacity measured in HILIC for GU 8–9 and GU 11–12. The maximum peak capacity for 1.7 μm column was achieved at flow rate between 0.3 and 0.5 mL/min. 1.7 μm sorbent was BEH Glycan column and 3.0 μm was a research batch of BEH sorbent. 2.1 mm × 50 mm column was used.

Comparison of the peak capacity of columns packed with 1.7 and 3.0 μm sorbent is shown in Fig. 2. As expected, columns packed with 3.0 μm particles have lower peak capacity. The theoretically predicted peak capacity gain for 1.7 μm versus 3.0 μm columns is 33% (calculated as a ratio of square root of *N* for both columns). We have experimentally observed ~38% better peak capacity for the 1.7 μm versus 3.0 μm HILIC column.

The peak capacity curves in Fig. 2 represent guidelines for further separation optimization for HILIC UPLC columns. While it is advisable to operate at flow rates near the optimal peak capacity, it is important to understand that the peak volumes increase with the increase flow rate. As a consequence, the detection sensitivity decreases [21]. Elevated column temperature was expected to further improve the peak capacity by improving column efficiency and enhancing mass transfer in the stationary phase for relatively large MW biopolymers (however, separation selectivity can be affected when altering separation temperature). When increasing column temperature from 40 to 60 °C, we observed an 11% increase in peak capacity (measured for 1.7 μm column, supplemental Fig. 1).

The side-by-side comparison of UPLC and HPLC columns separation performance is shown in Fig. 3 for fetuin 2-AB labeled glycans. The sample contains multiply sialylated glycans with positional isomers, which are often difficult to separate in conventional HPLC approach. The improvements in separation are mainly due to the higher peak capacity of UPLC column. Fig. 3 reveals that TSKgel Amide-80 column has comparable selectivity (at least under the separation conditions used in the experiment). This is not greatly surprising, since both HILIC columns are packed with amide type sorbents.

The additional data on column performance comparison are provided as supplemental material. Supplemental Figs. 2 and 3 illustrate enhanced separations of G1F isomers from IgG, and linkage isomers A3G3 from fetuin using 1.7 μm BEH Glycan UPLC column. The gradient slope for both UPLC and HPLC experiments was maintained constant by adjusting the flow rate (due to the column dimension differences, 2.1 mm × 150 mm versus 2.0 mm × 150 mm; see figure captions). The identification of glycans was confirmed in UPLC experiment using ESI-MS connected serially after the fluorescent detector. The peaks in HPLC chromatograms were labeled based on the analogy with UPLC-FL data. The details on LC–MS setup are described in Section 3.4.

The peak capacity, albeit important, is only a part of the separation optimization. Separation selectivity is equally important for resolution. The effects of initial gradient strength, gradient slope, and separation temperature on glycan separation selectivity will be discussed in Section 3.2.

3.2. *Optmization of IgG glycans separation*

Glycans released from glycoproteins are often complex mixtures, and difficult to resolve even with high peak capacity HILIC columns. We selected the 2-AB labeled N-linked glycans released from human IgG purchased from ProZyme as a relevant sample for further study.

The impact of initial gradient strength on separation selectivity is shown in Fig. 4. We chose a 2.1 mm × 50 mm, 1.7 μm HILIC column with the goal to achieve peak resolution in a relatively short analysis time. The gradient steepness was fixed to Δ10% MeCN

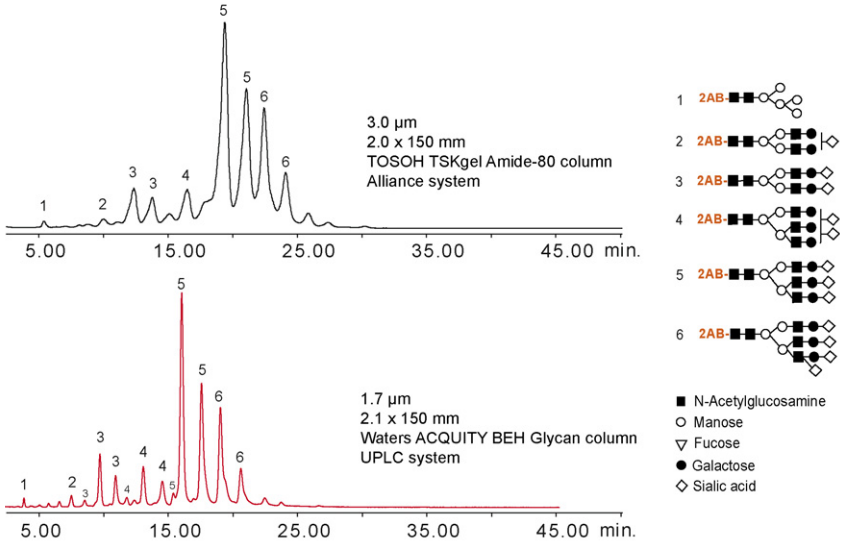

Fig. 3. Glycan separation comparison in 3.0 µm HPLC column and 1.7 µm sorbent. 10 pmol of fetuin 2-AB labeled glycans was separated using 3.0 µm TSKgel Amide-80 column in Alliance 2695 (top) and 1.7 µm BEH Glycan column in UPLC system (bottom). Sialylated biantennary and triantennary glycans including positional isomers were baseline resolved using 1.7 µm column in UPLC system in 45 min gradient time. The UPLC separation was done in gradient 65–55% B in 45 min at 0.5 mL/min using 2.1 mm × 150 mm and the HPLC separation was done in 65–55% B in 50 min at 0.45 mL/min using 2.0 mm × 150 mm. The column temperature was at 40 °C on both runs. The peaks labeled with same numbers indicate the isomers.

in 15 min (0.667% B/min) to eliminate gradient slope effects as a variable.

The changes in resolution for highlighted peaks at different initial gradient strengths are illustrated in Fig. 4. Given gradient condition in 72–62% B leads to better resolution in peaks 3 and 4 as well as peaks 7 and 8. Under the other gradients such as 75–65 and 70–60% B, peaks 3 and 4 were resolved better at higher initial organic concentration, however peaks 7 and 8 were separated better at lower initial organic strength. Peak identity was confirmed by LC/MS (see Section 3.4).

Similar behavior was also observed when changing the flow rate (which affects the gradient slope) as shown in Fig. 5. The observed changes in resolution are either positive (peak 8 at 0.2 mL/min and peak 13 at 0.8 mL/min) or negative (peak 13 at 0.2 mL/min and peak 8 at 0.8 mL/min). Increasing the flow rate leads to shallower gradients and changes in separation selectivity.

Changes of separation selectivity caused by gradient slope or initial gradient strength are well known. Snyder and Dolan use terminology such as "*irregular sample*" (separation selectivity changes with gradient conditions, e.g. in peptide mapping applications [26]) or "*regular sample*" (relative retention does not change with gradient, e.g. for homooligomers [27], such as dextran ladder) [28]. Figs. 4 and 5 indicate that glycan samples fall within the definition of *irregular sample* [28].

The experiment in Fig. 4 clearly illustrates that one does not have complete freedom to vary chromatographic conditions in order to achieve the optimum peak capacity. In fact, the flow rate may be used as one of the optimization parameters. We selected the developed HILIC method (72–62% MeCN in 15 min at flow rate of 0.4 mL/min) as a baseline for further separation optimization using longer columns (Section 3.3).

3.3. *Selection of column length and analysis time*

The gradient method developed for IgG glycans separation with a 50 mm long column (72–62% MeCN, 0.4 mL/min) was adapted to 100 and 150 mm columns. The goal of the experiment is to further enhance the glycan resolution by using columns with greater efficiency.

The method transfer between 50 and 100 or 150 mm long columns was shown in Fig. 6. In order to maintain constant separation selectivity, one needs to maintain the same gradient slope

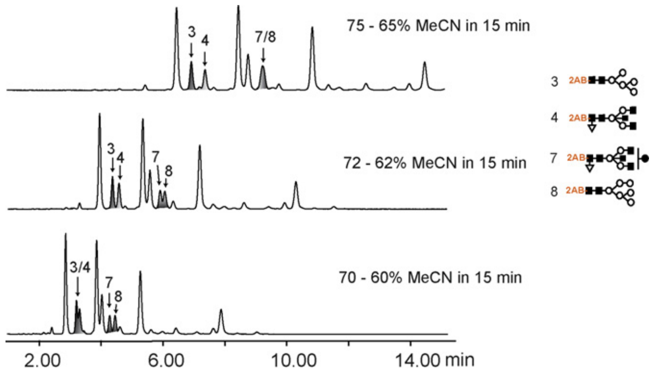

Fig. 4. Resolution comparison in initial organic strength change in HILIC gradient. 2-AB labeled glycans from IgG were separated at constant gradient slope in fixed gradient time, 15 min at flow rate 0.4 mL/min using a 2.1 mm × 50 mm column. The highlighted peaks in 72–62% B were efficiently separated while peaks 7 and 8 in 75–65% B and peaks 3 and 4 in 70–60% B were not resolved.

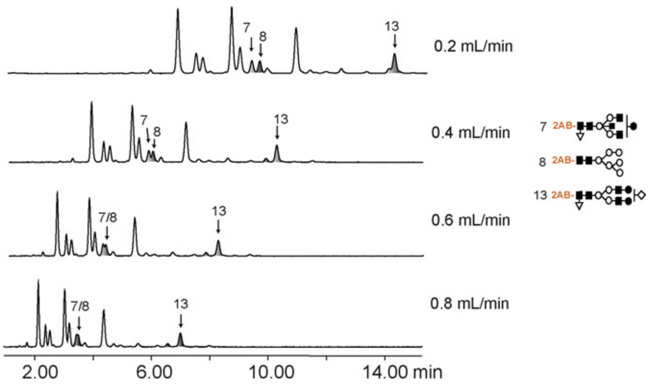

Fig. 5. Resolution comparison at various flow rates in HILIC gradient. 2-AB labeled glycans from IgG were run in 72–62% MeCN in 15 min using a 2.1 mm × 50 mm column. The highlighted peaks at 0.4 mL/min were efficiently separated while peaks 7 and 8 at 0.8 mL/min and peak 13 at 0.2 mL/min were not resolved.

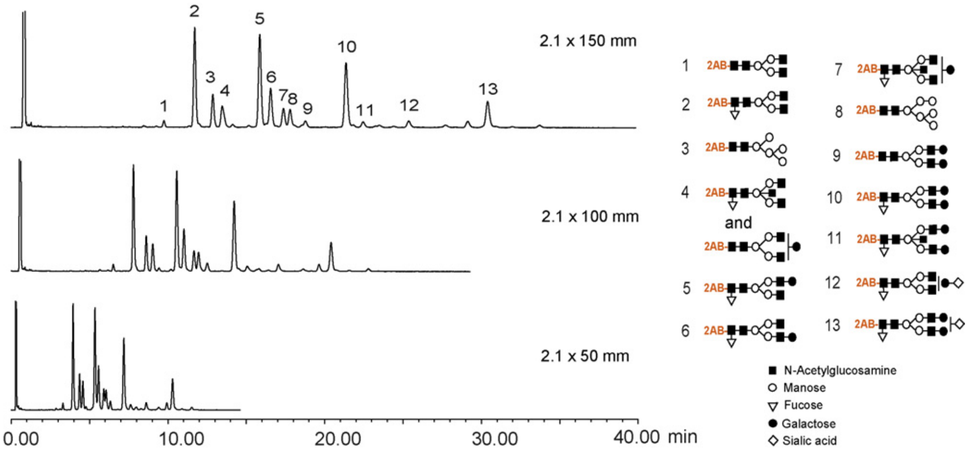

Fig. 6. 5 pmol of 2-AB labeled glycans released from human IgG. Condition: 1.7 μm sorbent packed in 2.1 × 50, 100, and 150 mm. The gradient condition was in 72–62% MeCN in 15, 30, 45 min gradient time, respectively, at flow rate 0.4 mL/min, and column temperature was 60 °C. The mobile phase A and B were prepared with 100 mM ammonium formate pH 4.5 and 100% MeCN, respectively.

s, defined by Eq. (2):

$$s = \Delta c \frac{t_0}{t_g} \quad (2)$$

Symbol t_0 represents the retention time of an unretained component, t_g is gradient time, and Δc is gradient span (for the case shown in Fig. 6, $\Delta c = 0.72–0.62 = 0.1$). Since t_0 increases with the column length, the gradient time t_g needs to be scaled proportionally in order to maintain constant gradient slope *s*.

It is shown that the separation selectivity at constant gradient slope is not altered; the gains in resolution are driven solely by the greater peak capacity (efficiency) of longer columns. The improved separation is achieved at the expense of gradient time. Improvements in resolution are most apparent for the 2.1 mm × 150 mm, 1.7 μm column (Fig. 6). Baseline resolution of most glycans was achieved, including the structural isomers (peaks 5 and 6) and the glycan pairs 3/4 and 7/8.

In addition, 100 or even 50 mm long columns provided a reasonable separation for critical pairs. This is consistent with the theory, which predicts that the peak capacity gains are proportional to the square root of the column efficiency *N* [21,22]. When transferring the method from 50 to 100 and 150 mm columns using a constant gradient slope, one achieves only ~41% or 73% peak capacity improvements, respectively (for 100 and 150 mm columns). In other words, despite using three times longer column and gradient time, the resolution gains are rather moderate. If one wishes to perform fast and efficient separations, it is beneficial to use short columns packed with a small particle size sorbent. The high resolution in fast separation using 1.7 μm BEH Glycan column in 2.1 mm × 150 mm is demonstrated for 2-AB labeled RNase B glycans (Supplemental Fig. 4). High mannose glycans including three isomers of mannose 7 were resolved under 30 min gradient from 70 to 60% B and a flow rate of 0.4 mL/min.

3.4. Glycan assignment with LC–MS

MS detection is useful for identification of glycans, but MS alone does not distinguish isomeric glycans with the same mass. We have implemented ESI-MS detection of glycans, connecting QTof type of mass spectrometer serially after fluorescent detector. The main

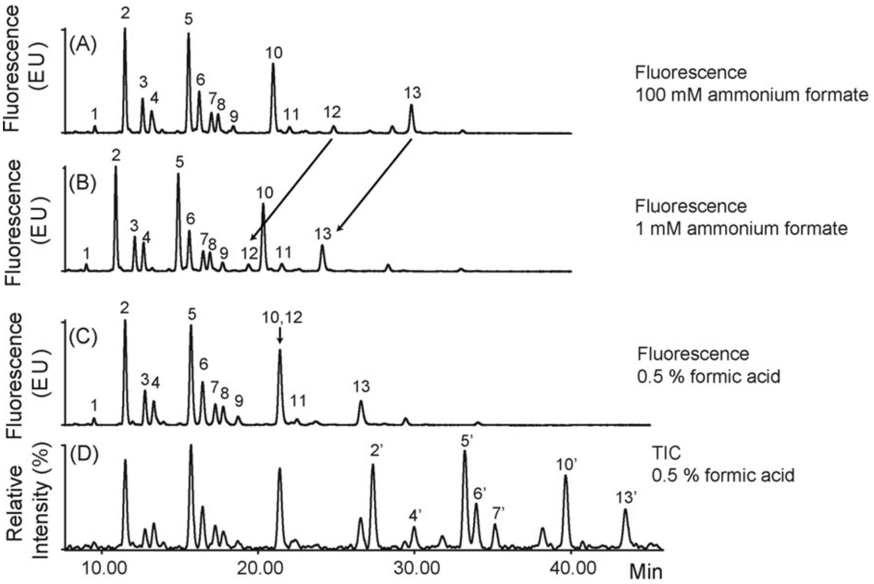

Fig. 7. 20 pmol of 2-AB labeled IgG glycans in UPLC-FL-ESI-MS. The chromatograms were compared in 100 mM (A), 1 mM (B) of ammonium formate pH 4.5 and 0.5% formic acid in FL (C) and in TIC (D). The selectivity of sialic acid glycans were changed while the neutral glycans retained less change in retention time. The run condition is same as described in Fig. 5 except the mobile phase conditions. 2.1 mm × 150 mm column was used. (See Fig. 6 for peak labeling.)

challenge of such an approach is the ionization suppression of glycans in MS caused by 100 mM ammonium formate buffer present in the mobile phase.

Due to the high ionic strength mobile phase (conditions shown in Fig. 7), we obtained sufficient MS signal only when scaling up the injection from sub-picomole level to 20 pmol of IgG glycan sample on column. The MS signal improved three to five times upon reducing ammonium formate concentration to 1 mM, or when using 0.1–0.5% formic acid (Supplemental Fig. 5). The peak shape and the peak capacity were maintained with these MS-friendly mobile phases. However, we observed moderate shift of the retention times for acidic glycans containing sialic acid residues (peaks 12 and 13 in Fig. 7B), while the separation selectivity of neutral glycans was not affected. This phenomenon has been reported earlier for amide HILIC columns [19]. We speculate that the retention of charged glycans at lower buffer concentration is reduced due to the ion repulsion from the residual negative charges present on the amide sorbent surface. When using high ionic strength mobile phases (e.g. 100 mM buffers), the ionic contribution to the separation is insignificant.

A total ion chromatogram (TIC) of 2-AB labeled IgG glycans analyzed with 0.5% formic acid in the mobile phase is shown in Fig. 7D. Acquired MS data confirmed the identity of the glycans purchased from manufacturer. Interestingly, the TIC MS chromatogram reveals the presence of additional peaks (peak # 2′–13′) related to 2-AB labeled glycans. These peaks do not give fluorescent signal at 330 nm excitation and 420 nm emission wavelengths (compare Fig. 7C and D).

Further investigation of MS data reveals that the peaks 2′–13′ have 15 Da mass shift from the masses of 2-AB labeled glycans, peaks 2–13. It appears that the sample contains a series of labeled glycans with a net loss of 15 Da from the 2-AB label, resulting in the diminished fluorescence. Since the labeled glycans were not detected in FL, we suspect the mass shift may be the net loss of N_1H_1 from the 2-AB label. This observation was confirmed by MALDI MS and MS/MS of the same sample, neither suggesting that neither the sample handling, nor HILIC chromatographic conditions are responsible for producing these by-products in the commercially available sample. The ability to detect and characterize unknown components in the glycan samples highlights the importance of LC–MS detection.

4. Conclusion

In this work, high resolution of 2-AB labeled glycans with an UPLC Glycan column was demonstrated. It was demonstrated that the optimal peak capacity for the 1.7 μm glycan column was observed at flow rates between 0.3 and 0.5 mL/min. The optimization parameters such as initial gradient condition, column temperature, and the gradient slope by adjusting the flow rate at constant gradient span can be used during HILIC method development. It was also shown that glycans behave as irregular samples, therefore separation selectivity changes with gradient conditions, similarly as known for peptides and other molecules. In HILIC mode, the peak capacity was improved with smaller particle size sorbent and longer columns, which agrees with theoretically predicted values, resulting in greater resolution. Finally, method transfer guidelines provide gains for rapid HILIC glycan analyses with enhanced resolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.12.013.

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