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Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis

Michael Melmer[a,*], Thomas Stangler[a], Andreas Premstaller[a], Wolfgang Lindner[b]

[a] Sandoz GmbH, Biochemiestrasse 10, 6250 Kundl, Austria
[b] University of Vienna, Waehringerstrasse 38, 1090 Vienna, Austria

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ABSTRACT

Hydrophilic-interaction liquid chromatography (HILIC), reversed-phase chromatography (RPC) and porous graphitic carbon (PGC) chromatography are typically applied for liquid chromatographic separations of protein N-glycans. Hence the performances of these chromatography modes for the separation of fluorescently labeled standard glycan samples (monoclonal antibody, fetuin, ribonuclease-B) covering high-mannose and a broad range of complex type glycans were investigated. In RPC the retention of sialylated glycans was enhanced by adding an ion-pairing agent to the mobile phase, resulting in improved peak shapes for sialylated glycans compared to methods recently reported in literature. For ion pairing RPC (IP-RPC) and HILIC ultra-high performance stationary phases were utilized to maximize the peak capacity and thus the resolution. But due to the shallow gradient in RPC the peak capacity was lower than on PGC. Retention times in HILIC and IP-RPC could be correlated to the monosaccharide compositions of the glycans by multiple linear regression, whereas no adequate model was obtained for PGC chromatography, indicating the significance of the three-dimensional structure of the analytes for retention in this method. Generally low correlations were observed between the chromatography methods, indicating their orthogonality. The high selectivities, as well as the commercial availability of ultra-high performance stationary phases render HILIC the chromatography method of choice for the analysis of glycans. Even though for complete characterization of complex glycan samples a combination of chromatography methods may be necessary.

1. Introduction

Protein glycosylation has implications for a variety of biological functions, e.g. cell–cell signaling, protein stability and solubility and affinity to target molecule. The glycan profile is particularly relevant for therapeutic proteins, because it impacts efficacy and safety of the product [1–3]. Thus, glycans of biopharmaceuticals must be characterized and also monitored during production, requiring both, in-depth characterization methods and fast profiling methods.

Chromatographic analysis of glycans is typically accomplished utilizing hydrophilic-interaction chromatography (HILIC), but may also be performed on conventional reversed-phase or porous graphitic carbon (PGC) stationary phases. The glycans are typically labeled with a fluorescence dye prior to their analysis. In reversed-phase chromatography (RPC) labeling with an aromatic tag is mandatory to generate retention of these highly hydrophilic analytes. In contrast PGC is typically employed also for reduced glycans, which are detected by mass spectrometry (MS). This fact is particularly remarkable, because interferences of the separation of glycans on PGC with the high-voltage of the electrospray ionization source were reported [4,5]. Nevertheless PGC is also capable of separating fluorescence labeled glycans. This aromatic label may then be utilized to introduce isotope tags for mass spectrometric detection [6].

In HILIC the fluorescent labeled glycans are retained according to the number and accessibility of polar groups. Several commercially available phases possess charged moieties additionally providing coulomb interactions [7]. Retention is generally correlated to size of the glycans with certain selectivities for isomers. Due to the high fraction of organic solvent in HILIC (usually acetonitrile) the column back pressure is comparably low, allowing for the use of ultra-high-performance liquid chromatography (UHPLC) columns with standard HPLC equipment at least at elevated temperature. Furthermore, remarkable resolution of a highly sialylated glycan sample was achieved on a weak anion-exchange column operated in HILIC mode, illustrating the potential of combinations of retention mechanisms [8].

* Corresponding author. Tel.: +43 5338 200 2060; fax: +43 5338 200 9345.
E-mail addresses: michael.melmer@sandoz.com (M. Melmer), thomas.stangler@sandoz.com (T. Stangler), andreas.premstaller@sandoz.com (A. Premstaller), wolfgang.lindner@univie.ac.at (W. Lindner).

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In RPC the glycans elute in groups according to their structure elements [9]. The elution order may depend on specific column properties, e.g. end-capping or silanol activity, because retention order varies between ODS columns of different brands [10]. Furthermore retentivity of fluorescently labeled glycans on conventional RP columns is quite low thus limiting the fraction of organic solvent in the mobile phase.

Porous graphitic carbon (PGC) is employed for glycan analysis mainly in the academic field. This stationary phase solely consists of graphite type carbon and offers remarkable selectivities for isomeric glycans and increased retention particularly for charged glycans [11,12]. The strong adsorption of polar analytes on PGC compared to conventional reversed-phases even allows for the analysis of native and reduced oligosaccharides [13,14]. In previous experiments we identified the nature of the organic modifier, the column temperature and the redox state of the PGC material as important parameters impacting retention of oligosaccharides [15,16].

The development of UHPLC equipment significantly increases the peak capacities achievable in liquid chromatography and enables for shorter analysis times. For several years the range of UHPLC columns was restricted to RPC, which have been investigated for high-throughput analysis of fluorescently labeled immunoglobulin G (IgG) glycans [10]. But recently a HILIC type UHPLC stationary phase dedicated to glycan analysis has become available, which was tested for RNase-B, fetuin and IgG glycans [17].

In this contribution we report on the investigation of HILIC, ion pairing RPC (IP-RPC) and PGC chromatography for the analysis of 2-aminobenzamide (2-AB) labeled glycans. The retention times of 2-AB labeled glycans from fetuin, RNase-B and a mAb in HILIC, IP-RPC and PGC chromatography, respectively, were determined applying linear gradients. Based on this broad data set the retention properties of the stationary phases regarding protein N-glycans were correlated to the monosaccharide compositions by multiple linear regression (MLR). This evaluation allows to quantitatively assess the contributions of the different monosaccharides to the retention in each investigated chromatography mode. Furthermore the goodness of fit indicates whether retention is determined solely by the monosaccharide composition or it is impacted by further parameters.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were at least analytical grade. RNase-B was purchased from Worthington Biochemical (Lakewood, NJ, USA), fetuin was ordered from Sigma (Steinheim, Germany). The mAb was obtained from in-house development at Sandoz (Sandoz, Kundl, Austria). PNGase-F and neuraminidase were ordered from Roche (Roche Diagnostics, Vienna, Austria). Water was purified by a MilliQ® system (Millipore; Billerica, MA, USA). Gradient grade acetonitrile and methanol (p.a.) were purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation

The glycans of a mAb were released by incubation with PNGase-F at 37 °C in 15 mM Tris/HCl pH 7.0 over night. Afterwards the glycans were separated from the proteins by ultrafiltration using Microcon® YM-30 centrifugal filter devices. The reducing end was labeled with 50 mg/mL 2-aminobenzamide and 63 mg/mL $Na[BH_3(CN)]$ in dimethylsulfoxide/acetic acid 7:3 at 37 °C over night. The excess label was depleted by gel filtration on PD MiniTrap™ Sephadex® G-10 column. Fetuin and RNase-B were denaturated in 6 M guanidine chloride at 80 °C for 2 h prior to the PNGase-F digest.

An aliquot of fetuin glycans was treated with neuraminidase, according to the instructions of the manufacturer, to remove the sialic acids. The resulting sample is termed “asialo fetuin” within this publication.

2.3. Instrumentation

Fluorescence chromatograms were recorded on an Agilent 1200 SL system, consisting of a binary pump, a vacuum degasser, an autosampler, a column thermostat and a fluorescence detector. The excitation wavelength was 250 nm and the emission was recorded at 428 nm.

2.4. Hydrophilic interaction chromatography

The glycan samples from fetuin, asialo fetuin and RNase-B were fractionated on a Waters Acquity UPLC® BEH Glycan column (2.1 mm i.d., 150 mm length) packed with 1.7 μm particles. The temperature of the column thermostat was set to 60 °C. Solvent A was acetonitrile and solvent B was 150 mM formic acid titrated to pH 4.4 with ammonia solution (25%). For fractionation of fetuin glycans a linear gradient starting with 37% B and reaching 42% B after 40 min was applied. Asialo fetuin and RNase-B glycans were fractionated utilizing a linear gradient from 30% B to 45% B within 40 min. The flow was 0.5 mL/min. Fractions were collected manually.

Retention times of the individual glycans were determined by re-injection on the same column. The method started with 30% B, which was increases by 0.5%/min. The column temperature was maintained at 60 °C. Retention times determined by re-injection were employed for data analysis.

The monosaccharide compositions of the glycans were determined by MS analysis using a LIT-Orbitrap® instrument (Thermo Electron). This type of mass spectrometer provides accuracies typically below 10 ppm, which allows for reliable determination of the monosaccharide composition.

For analysis by IP-RP and PGC chromatography the fractions were concentrated in a centrifugal evaporator. For re-chromatography the fractions were diluted with water 1:10. Samples exhibiting unsatisfactory peak shape when re-chromatographed were diluted with water 1:50.

2.5. Ion pairing reversed-phase chromatography

For the reversed-phase separation a Waters Acquity® UPLC® BEH300 C18 column (2.1 mm i.d., 100 mm length) packed with 1.7 μm particles was utilized. As for all chromatography methods, a linear gradient was developed to achieve elution of all glycans within approximately 30 min. Mobile phase A contained 20 mM diethylamine (DEA) as ion pairing agent and 50 mM formic acid in water. Mobile phase B was 20 mM DEA and 50 mM formic acid in 25% methanol and 75% water. The gradient started with 10% B, which was raised to 20% B within 40 min. The flow rate was 350 μL/min. The column was maintained at 60 °C.

2.6. Porous graphitic carbon chromatography

A Thermo Hypercarb™ column (3 mm i.d., 100 mm length) packed with 3 μm particles was used for PGC chromatography. Mobile phase A contained 100 mM trifluoracetic acid (TFA) and 50 mM ammonia in water. Mobile phase B contained 100 mM TFA and 50 mM formic acid in 55.6% acetonitrile and 44.4% water. A linear gradient from 45% to 100% B within 30 min was applied. The