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Short communication

Separation of monosaccharides by hydrophilic interaction chromatography with evaporative light scattering detection

Göran Karlsson*, Stefan Winge, Helena Sandberg

Octapharma AB, SE-11275 Stockholm, Sweden

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Abstract

Hydrophilic interaction liquid chromatography (HILIC) was used to separate monosaccharides that are common in N-linked oligosaccharides in glycoproteins and other compounds. A TSKgel Amide-80 column was eluted with 82% acetonitrile, in 5 mM ammonium formate (pH 5.5). Column temperature was 60 °C and evaporative light scattering was used for detection (ELSD). With this method, L-fucose, D-galactose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetylneuraminic acid, and D-glucuronic acid were separated, with detection limits of 0.3–0.5 μg for each monosaccharide, and intermediate precisions were 3–6% RSD ($n = 6$).

Keywords: Hydrophilic interaction liquid chromatography (HILIC); Monosaccharides; Evaporative light scattering detection (ELSD)

1. Introduction

The analysis of monosaccharides and more complex carbohydrates is often performed by column liquid chromatography (LC) techniques. Normal-phase liquid chromatography (NPLC), using amino-bonded silica columns, has been widely used for many years [1]. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) is a high-resolution technique for analyzing carbohydrates [2]. Following derivatization of the reducing end of the monosaccharides, where a hydrophobic chromophore is attached, reversed-phase liquid chromatography (RPLC) can be used for separation [3]. Ligand-exchange chromatography (LEC), based on the formation of a complex between hydroxyl groups of carbohydrates and multivalent metal ions, such as Pb^{2+}, bound to a cation exchange chromatography column, has been used for the separation of monosaccharides [4]. Many other techniques have also been used for the separation of monosaccharides, e.g. Concanavalin A affinity chromatography [5], fluorophore-assisted carbohydrate electrophoresis (FACE) [6], gas chromatography/mass spectrometry (GC/MS) [7], and capillary electrophoresis (CE) [8]. Cyclodextrin-bonded silica gel columns [9] and supercritical fluid chromatography (SFC) [10] have also been used for carbohydrate analysis.

Hydrophilic interaction liquid chromatography (HILIC) may be described as a variant of normal-phase chromatography, where a hydrophilic stationary phase is used in combination with a mostly organic mobile phase, and elution is usually performed by increasing the water concentration. HILIC was introduced by Alpert [11] and has been used to analyze different types of carbohydrates, including monosaccharides, disaccharides and oligosaccharides [12–15]. Monosaccharides that are common in glycoproteins, however, have not been separated by HILIC. Many other polar compounds, e.g. peptides and amino acids have also been analyzed by HILIC [11]. Direct detection of carbohydrates by UV absorbance or fluorescence is usually not possible and refractive index (RI) detectors therefore are commonly used. Evaporative light scattering detection (ELSD) is a semi-universal HPLC detector, widely used to detect non-volatile compounds, such as carbohydrates [1] and lipids [16]. In contrast to RI detection, ELSD may also be used with gradient elution.

In this work, HILIC was used with ELSD detection, to separate several monosaccharides that are common in N-linked oligosaccharides in glycoproteins and other compounds.

* Corresponding author.
E-mail address: goran.karlsson@octapharma.se (G. Karlsson).

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2. Materials and methods

Ammonium formate, D-glucose, D-mannose, D-galactose, L-fucose, *N*-acetyl-D-galactosamine, *N*-acetylneuraminic acid, and D-glucuronic acid (all of analytical grade) were obtained from Sigma (Munich, Germany), and *N*-acetyl-D-glucosamine (>99%) was from Alfa Aesar (Karlsruhe, Germany). Acetonitrile (far UV) was obtained from Labscan (Dublin, Ireland), formic acid was from BDH (Poole, UK), and water was taken from a Milli-Q apparatus (Millipore, Molsheim, France).

2.1. Separation of monosaccharides by HILIC

A Waters Alliance 2695 HPLC system was used together with a 996 PDA UV detector, and Millennium as the controlling software (Waters, Milford, MA, USA). The main detection of monosaccharides was carried out using an ELSD detector, Sedex 75, from Sedere (Vitry sur Seine, France), coupled in-line after the UV detector. Nitrogen was used as the ELSD nebulizer gas (3.5 bar), at a temperature of 40 °C, and the gain was set to 8. The HPLC system included a carbamoyl-silica HILIC column (TSKgel Amide-80, 5 μm, 80 Å, 250 mm × 4.6 mm I.D., from Tosoh, Stuttgart, Germany). The column was run at 60 °C and the mobile phase used for isocratic elution contained 82% acetonitrile, in 5 mM ammonium formate (adjusted to pH 5.5 with formic acid). Standard curves from a mixture of L-fucose, D-galactose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetylneuraminic acid, and glucuronic acid (40, 60, 80 and 100 μg/mL of each monosaccharide), with a log-log linear curve fit, was used. All samples were injected in 90% acetonitrile. The flow-rate was 1.0 mL/min and injection volumes were 50 μL.

3. Results and discussion

The optimized HILIC method presented here gave separated peaks for all common N-linked sugar residues (L-fucose, D-galactose, D-mannose, *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid) in mammalian glycoproteins (Fig. 1). In addition, D-glucuronic acid was also separated. The lowest resolution (R_S) obtained was between mannose and galactose, $R_S = 0.9$ at the 5 μg level, and was considered acceptable. The monosaccharide L-fucose (6-deoxy-L-galactose) gave the lowest retention on the polar HILIC column, probably because L-fucose does not have as many polar hydroxyl groups as does, for example, D-mannose or D-galactose. *N*-Acetylneuraminic acid (the most common sialic acid) and D-glucuronic acid, both containing several polar hydroxyl groups and one charged carboxyl group (at pH 5.5), gave the highest retention (Fig. 1).

During method development the column was initially run at 50 °C, using 82% acetonitrile, in 5 mM ammonium acetate, based on a previously described method [13]. In optimizing this method, ammonium acetate was replaced with ammonium formate because of a slightly higher response to the monosaccharides on the ELSD detector, when using the same detector settings. The temperature was also increased to 60 °C, because of the increased resolution and peak height (Fig. 1). An increased concentration of ammonium formate increased the retention times for *N*-acetylneuraminic acid and D-glucuronic acid. We speculate that this might be dependent on the increased formation of a complex between the carboxylic groups in the monosaccharides and the ammonium ions in the mobile phase, and that this complex is strongly bound to the column. The Tosoh manufacturer recommends pH 2.0–7.5 and 10–80 °C being used for this HILIC column, and, thus, a temperature of 60 °C in combination with pH 5.5 would be expected to give an acceptable column life time. The optimized method gave about three times lower detection limits than the initial conditions. At temperatures below 40 °C the reducing monosaccharides, e.g. L-fucose gave partially resolved double peaks (data not shown), likely due to α- and β-anomers formed by mutarotation [12,17]. An elevated temperature, increase of pH by addition of amino compounds, or increase in the concentration of acetonitrile have been shown to facilitate the collapse of anomer peaks, resulting in one distinctive single peak [12,17].

Six analyses were run, using separate standard curves of a mixture of all six monosaccharides. A reference sample, also containing a mixture of all six monosaccharides, was analyzed in all analyses and the intermediate precisions for this sample were 3–6% RSD for the six monosaccharides (Table 1). The suboptimal resolution between mannose and galactose, which gave a small peak overlap, may influence the results obtained for these carbohydrates. The mean linear correlation coefficients (from the six analyses) for the log-log standard curves (2–5 μg) were ≥0.99 and the limits of detection (S/N > 2) were 0.3–0.5 μg for all monosaccharides (Table 1). These detection limits are at the same level as reported previously, where NPLC with ELSD detection was used for carbohydrate analysis [1].

The selectivity of the HILIC method was investigated and D-glucose and *N*-acetyl-D-galactosamine were found to coelute with D-galactose and *N*-acetyl-D-glucosamine, respectively (Fig. 1). Coelution of glucose and galactose using the Amide-80 HILIC column has previous been reported [13]. However, with respect to the analysis of carbohydrate composition in glycoproteins, D-glucose is included in N-linked oligosaccharides during biosynthesis, but is not commonly present in mature glycoproteins, and *N*-acetyl-D-galactosamine is a sugar residue of O-linked carbohydrate chains in proteins. The method

Table 1
HILIC analysis of monosaccharides

	Fuc	GlcNAc	Man	Gal	Neu5Ac	GlcA
Precision						
Mean (μg/mL)	49	49	49	48	51	51
RSD (%)	4	3	4	6	5	6
Correlation coefficient (r)	1.00	1.00	0.99	1.00	0.99	0.99
LOD (μg)	0.3	0.3	0.3	0.3	0.5	0.5

The nominal concentrations of all six monosaccharides in the sample used for determination of precision were 50.0 μg/mL. Precision and correlation coefficient mean values are based on six separate analyses. Limit of detection (LOD) indicates S/N > 2.

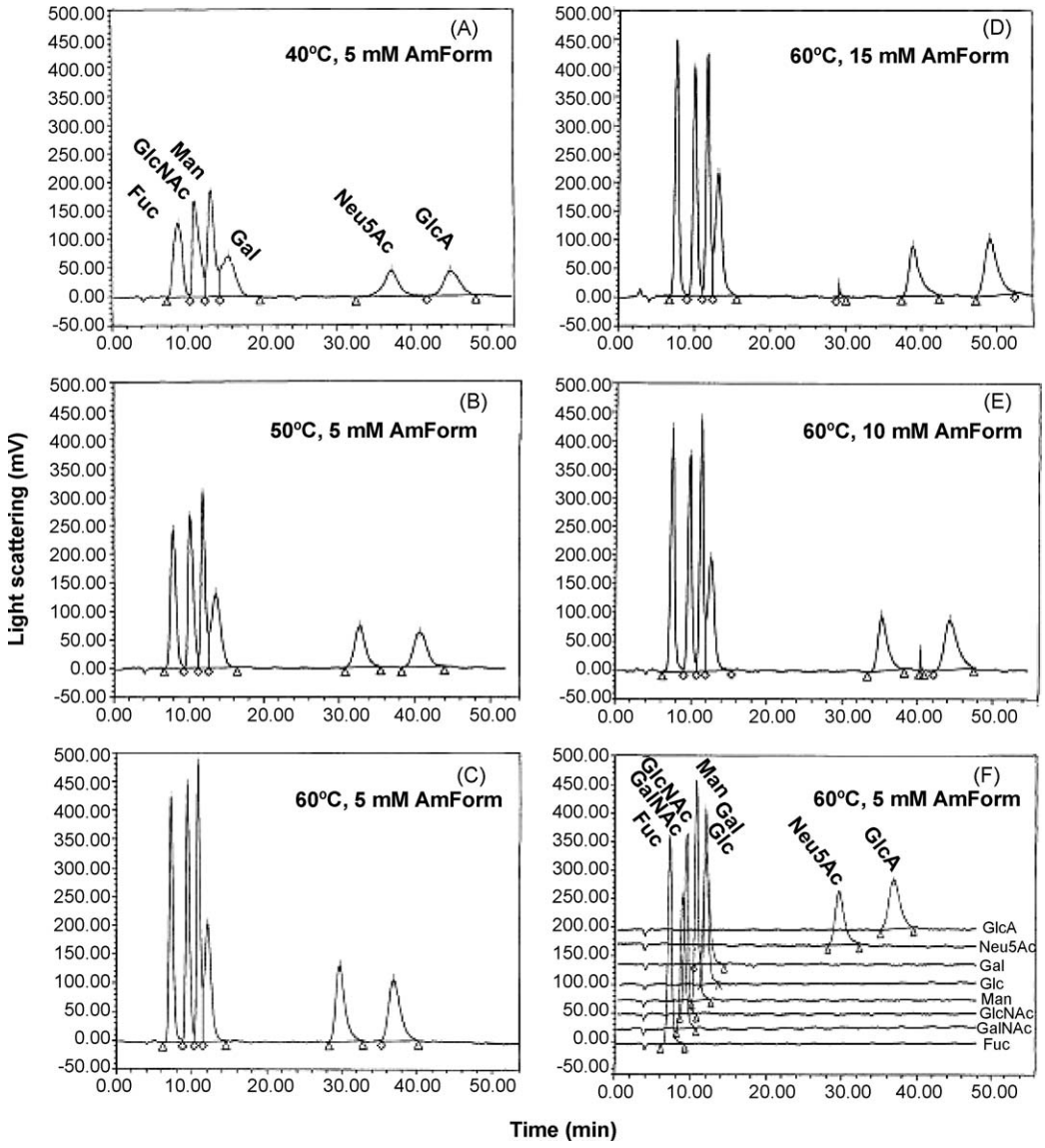

Fig. 1. Separation of monosaccharides by HILIC. Influence of temperature and ammonium formate (AmForm, pH 5.5) concentration on the separation is shown in (A)–(E), and the selectivity results are given in (F). A TSKgel Amide-80 column, and acetonitrile, 82%, was used in all experiments. (A) 40 °C, 5 mM AmForm; (B) 50 °C, 5 mM AmForm; (C) 60 °C, 5 mM AmForml (D) 60 °C, 15 mM AmForm; (E) 60 °C, 10 mM AmForm; and (F) 60 °C, 5 mM AmForm. Mixtures of all six monosaccharides [L-fucose (Fuc), D-mannose (Man), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetylneuraminic acid (Neu5Ac), and D-glucuronic acid (GlcA)], 5 μg of each, were injected in (A)–(E). In F, showing the overlay of eight chromatograms, the same six monosaccharides, and, in addition also *N*-acetyl-D-galactosamine (GalNAc) and D-glucose (Glc), were analyzed separately. The conditions used in (C) and (F) represent the optimized method. Detection was performed by ELSD. See Section 2 for further details.

presented here can be used to analyze N-linked sugar residues of glycoproteins, preferentially after an initially specific enzymatic cleavage of N-linked oligosaccharides. The method, however, is not optimal for analyzing monosaccharides emanating from a mix of both N- and O-linked oligosaccharides of proteins, because *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine coelute. The method may also be used to separate monosaccharides, e.g. D-glucuronic acid and *N*-acetyl-D-glucosamine, from other types of carbohydrate-containing substances, such as glycosaminoglycans (GAGs).

The HILIC column (TSKgel Amide-80) used here has previously been used to separate monosaccharides [13], and in combination with MS [15]. In these papers, however, or in other HILIC studies, of which we are aware, the monosaccharides that are commonly present in glycoproteins were not analyzed. One major advantage of HILIC in the analysis of carbohydrates, in comparison with HPAEC, is that it is easily combined with the direct detection by an ELSD or an MS instrument, due to the volatility of the HILIC mobile phases. However, HPAEC has also been successfully used in combination with on-line MS detection, using ion suppression devices [18].

In conclusion, an isocratic HILIC–ELSD method was developed, to separate L-fucose, D-galactose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetylneuraminic acid, and D-glucuronic acid, with acceptable selectivity, intermediate precision, and linearity.

References

[1] Y. Wei, M. Ding, J. Chromatogr. A 904 (2000) 113.
[2] C. Panagiotopoulos, R. Sempéré, R. Lafont, P. Kerhervé, J. Chromatogr. A 920 (2001) 13.
[3] H. Kwon, J. Kim, Anal. Biochem. 215 (1993) 243.
[4] M.H. Gey, K.K. Unger, Fresenius' J. Anal. Chem. 356 (1996) 488.
[5] F. Santori, J. Hubble, J. Chromatogr. A 1003 (2003) 123.
[6] A. Calabro, V.C. Hascall, R.J. Midura, Glycobiology 10 (2000) 283.
[7] L. Kenne, S. Stromberg, Carbohydr. Res. 198 (1990) 173.
[8] T. Soga, D.N. Heiger, Anal. Biochem. 261 (1998) 73.
[9] A. Berthod, S.S.C. Chang, J.P.S. Kullman, D.W. Armstrong, Talanta 47 (1998) 1001.
[10] B. Herbreteau, M. Lafosse, L. Morin-Allory, M. Dreux, J. Chromatogr. 505 (1990) 299.
[11] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[12] A.J. Alpert, M. Shukla, A.K. Shukla, L.R. Zieske, S.W. Yuen, M.A.J. Ferguson, A. Mehlert, M. Pauly, R. Orlando, J. Chromatogr. A 676 (1994) 191.
[13] H. Kakita, H. Kamishima, K. Komiya, Y. Kato, J. Chromatogr. A 961 (2002) 77.
[14] G. Karlsson, A.C. Hinz, S. Winge, J. Chromatogr. Sci. 42 (2004) 361.
[15] V.V. Tolstikov, O. Fiehn, Anal. Biochem. 301 (2002) 298.
[16] W.W. Christie, W.R. Morrison, J. Chromatogr. 436 (1988) 510.
[17] C. Brons, C. Oloeman, J. Chromatogr. 259 (1983) 79.
[18] H.A. Schols, M. Mutter, A.G. Voragen, W.M.A. Niessen, R.A. van der Hoeven, J. van der Greef, C. Bruggink, Carbohydr. Res. 261 (1994) 335.