

Combination of Two Hydrophilic Interaction Chromatography Methods That Facilitates Identification of 2-Aminobenzamide-Labeled Oligosaccharides

Göran Karlsson*, Elin Swerup, and Helena Sandberg

Octapharma AB, SE-11275 Stockholm, Sweden

Abstract

Hydrophilic interaction chromatography (HILIC) is used to separate 2-aminobenzamide- (2-AB) labeled *N*-linked oligosaccharides. The glycans of the model protein, bovine fetuin, are identified following comparison of elution patterns of seven 2-AB-labeled glycan standards, of which two are of the high-mannose type and five are of the complex type. The combination of two HILIC methods, using an Amide-80 column, having different resolutions and selectivities, markedly facilitates the identification of the fetuin glycans. These HILIC methods are suitable for obtaining glycan profiles of complex mixtures.

Introduction

Most proteins are glycosylated and the linked carbohydrates are important for various biological functions. In addition, sialylation is crucial for the serum half-life of glycoproteins (1). Thus, glycosylation must be analyzed and evaluated (e.g., in producing recombinant glycoproteins for pharmaceutical use). The analysis of *N*-linked glycans from glycoproteins is complicated due to the diversity of structures and the many possible isomers, and often requires a combination of analytical methods for the characterization (2,3).

For analyzing *N*-linked glycans, several techniques have been used, including high-performance liquid chromatography (HPLC), capillary electrophoresis (4), and fluorophore-assisted carbohydrate electrophoresis (5). The HPLC techniques are widely used for analysis and characterization of carbohydrates, with the most recognized HPLC method being high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). In HPAEC, sugar hydroxyl groups are ionized by a highly alkaline mobile phase, which promotes the binding of the carbohydrates to the anion exchange column, followed by elution, by, for example, using a sodium acetate gradient (6). This technique has been popular due to its high resolution and ability to detect carbohydrates by PAD without previous derivatization. Also, conventional anion-

exchange chromatography has been successfully used to separate non-, mono-, di-, tri-, and tetrasialic acid containing glycans, followed by further analysis of the collected fractions (2). Anion-exchange chromatography columns have also been used for separating glycans in a combined mode. In the implementation of this technique, the sample is injected on a column that has been equilibrated with a high concentration of organics and some water, and the elution is performed by simultaneously increasing both the water concentration and the ionic strength. This is thus a combination of normal-phase liquid chromatography (NPLC) and ion-exchange chromatography (IEX), the so-called NP-IEX, which has a very high resolution (7). Reversed-phase liquid chromatography (RPLC) separation of glycans is usually carried out after fluorescent and hydrophobic tags are attached (8). RPLC and NPLC, the latter including hydrophilic interaction chromatography (HILIC), are, in contrast to HPAEC and IEX, readily combined with an electrospray ionization interface (ESI) in a liquid chromatography-mass spectrometry system. Derivatization with fluorescent groups [e.g., 2-aminobenzamide (2-AB) or 2-anthranilic acid (2-AA)] is often performed to detect glycans at low concentrations (9). NPLC, using amino columns, have been used for a long time in analyzing oligosaccharides (10), and cyclodextrin-bonded silica gel columns have also been successfully used for this purpose (11).

HILIC is a variant of NPLC, where a hydrophilic stationary phase is combined with a mostly organic aqueous mobile phase and elution is performed by increasing the polarity of the mobile phase by increasing the water concentration. HILIC was introduced by Alpert in 1990 (12) and has been used for the analysis of different types of carbohydrates (13–16). When using HILIC for the analysis of glycans and other carbohydrates, in addition to a high concentration of acetonitrile and some water, various concentration of buffer, often ammonium acetate or ammonium formate, is usually added to the mobile phases (14,17–20). In developing new HILIC methods it is advantageous to have knowledge of the potential effect of the concentration of added buffer on the method's resolution and selectivity.

This work describes the use of HILIC for analysis of 2-AB-labeled *N*-linked oligosaccharides. The influence of different concentrations of ammonium acetate and different temperatures on

*Author to whom correspondence should be addressed: email goran.karlsson@octapharma.se.

the separation was investigated. A combination of two HILIC methods was found to greatly facilitate the identification of the glycans of the model protein, fetuin.

Experimental

Materials

Bovine fetuin was from Sigma-Aldrich (Steinheim, Germany). Peptide *N*-glycosidase F (PNGase F, also called *N*-glycanase), the 2-AB labeling kit, all seven glycan standards (Figure 1), and the solid-phase extraction cartridges (GlykoClean R and GlykoClean S) were obtained from ProZyme/Glyko (San Leandro, CA). The detergent RapiGest was from Waters (Milford, MA). Acetonitrile (Far UV) and methanol (HPLC grade) were from LabScan (Dublin, Ireland). Additional chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany) or from Sigma-Aldrich.

Preparation of 2-AB-labeled *N*-linked glycans

300 μ g fetuin was incubated in 0.1% (w/v) RapiGest, 50mM 2-mercaptoethanol, 50mM ammonium acetate, and 0.5mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, in a total volume of 48 μ L, for 5 min at 95°C. After cooling, 5 mIU PNGase F (2 μ L) was added to the solution, which was then incubated for 16 h at 37°C (21). A blank sample of fetuin, containing exactly the same components as described earlier, except for PNGase F, was also incubated. The efficiency of the deglycosylation of fetuin was checked, as previously described (22), with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, using a 12.5% polyacrylamide homogeneous PhastGel, from GE Healthcare (Uppsala, Sweden) (23), and then followed by silver staining (24). After the enzymatic deglycosylation, protein and detergent was removed by GlykoClean R, according to the manufacturer's description (25) and the eluate containing glycans and other hydrophilic compounds was dried by a SpeedVac centrifugal evaporator (Thermo Electron Corporation, Waltham, MA). The 2-AB labeling of glycans was followed by purification by the GlykoClean S cartridge, to remove surplus of reagents, and performed according to the description from ProZyme/Glyko (26–28). The 2-AB-labeled glycans were finally dried by the SpeedVac centrifugal evaporator. The purchased glycan standards were labeled with 2-AB and purified by GlykoClean S, as described earlier.

Hydrophilic interaction chromatography

A Waters Alliance 2695 HPLC system was used together with a 474 Fluorescence detector, and Empower as the controlling software. The 2-AB derivatives were excited at 330 nm and emission was measured at 420 nm by the fluorescence detector. The gain was set to 1000, and the slit widths were 18 nm. The separa-

tion of 2-AB-labeled oligosaccharides was performed on a TSKgel Amide-80 column (5 μ m, 80 Å, 250 \times 4.6 mm i.d., from Tosoh Biosep, Stuttgart, Germany). The purified and dried 2-AB-labeled glycans were reconstituted in 70% acetonitrile (ACN) and 30% water, containing 5mM ammonium acetate (pH 5.5, adjusted by acetic acid). Approximately 25 ng of each of the seven standard glycans were injected in 5 μ L. The flow rate was 1 mL/min. Four HILIC method variants (A–D) were used for the optimization. HILIC method A and HILIC method D were used with aqueous gradients (0–20 min 64% ACN, 20–50 min 64–52% ACN, 50–65 min 64% ACN) in the presence of 5mM ammonium acetate, pH 5.5, for elution. HILIC method B and HILIC method C were used with aqueous gradients (0–20 min 64% ACN, 20–60 min 64–52% ACN, 60–75 min 64% ACN) in the presence of 25 and 100mM ammonium acetate (pH 5.5), respectively, for elution. The separations were performed at 45°C, except for HILIC method D, which was performed at 30°C.

Results

In this work, bovine fetuin was used as a model glycoprotein for developing an HILIC method for glycan profiling. Fetuin has a total molecular weight of 48.4 kDa, 26% of which is carbohydrate. *N*-Linked oligosaccharides account for 80% of the carbohydrate. More than 23 distinct *N*-linked fetuin glycans, all of the complex type, have been characterized (29). The results from the SDS-PAGE indicated a complete deglycosylation of fetuin by PNGase F (results not shown). Seven purified glycan standards from ProZyme/Glyko were labeled with 2-AB and analyzed by HILIC. Two of these standards were of the high-mannose type and five were of the complex type (Figure 1).

The optimization of the HILIC method was done by analyzing

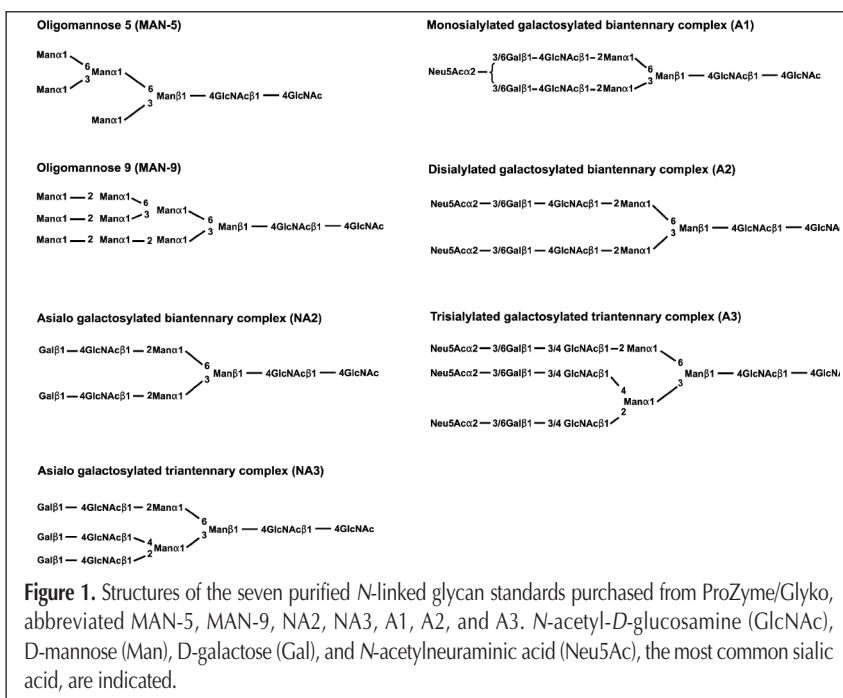


Figure 1. Structures of the seven purified *N*-linked glycan standards purchased from ProZyme/Glyko, abbreviated MAN-5, MAN-9, NA2, NA3, A1, A2, and A3. *N*-acetyl-*D*-glucosamine (GlcNAc), *D*-mannose (Man), *D*-galactose (Gal), and *N*-acetylneuraminic acid (Neu5Ac), the most common sialic acid, are indicated.

the mixture of the seven glycan standards at 30°C and 45°C, and using 5, 25, and 100mM ammonium acetate, with segmented gradients of increasing water in the acetonitrile/water mobile phase systems (Figure 2). The chromatograms from the blank sample of fetuin (with no PNGase F) gave a flat baseline in all HILIC methods (results not shown). Increasing the column temperature from 30°C to 45°C, when 5mM ammonium acetate was used, improved the resolution for the analysis of the mixture of the seven glycan standards, and an additional peak appeared at 12 min (Figure 2A and 2D). A column temperature of 60°C was also tested, but did not significantly improve the resolution (results not shown). The pH 5.5 of the mobile phase, and a temperature of 45°C were chosen to minimize the hydrolysis of sialic acid of the glycans and to promote stability of the column. HILIC methods A and B at 45°C, using 5 and 25mM ammonium acetate, respectively, showed the best resolution and peak height, while 100mM ammonium acetate (method C) in the gradient

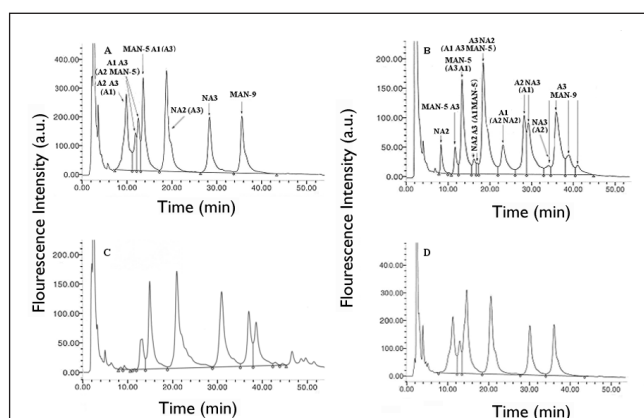


Figure 2. Optimization of the HILIC system, using acetonitrile, ammonium acetate (pH 5.5), and water for elution. A mixture of seven 2-AB-labeled glycan standards was separated by the Amide-80 column at different concentrations of ammonium acetate and at different temperatures. 5 mM ammonium acetate, 45°C (A); 25 mM ammonium acetate, 45°C (B); 100 mM ammonium acetate, 45°C (C); and 5 mM ammonium acetate, 30°C (D). A-D represent HILIC methods A-D, respectively. Two different scales were used for the response axis. Peaks labeled within the parentheses in the chromatograms were considered to represent minor components. See the Experimental section for further details.

Table I. Relative Content of N-linked Glycans in Bovine Fetuin. Results from this Study, Based on Both HILIC Method A and HILIC Method B, Compared to Literature Values.

Glycan	Results (%) from Green et al. (29)	Results (%) from this study
MAN-5, MAN-9	Not found	Not found
NA2, NA3	Not found	Not found
A1	1	5
A2	16	7
A3	54	79
A1 + A2	17	13*

* One peak included (at 22.3 min, with an area of approximately 1%), which eluted at the overlap in retention times for A1 and A2.

showed less resolution and lower peaks (Figure 2). Therefore, methods A and B were chosen for further studies.

The result from HILIC method A, from the analysis of the separate glycan standards and the fetuin glycans, is shown in Figure 3. The interpretation of the chromatograms from oligosaccharides derived from fetuin was performed by comparing the chromatograms with those obtained from the analyses of the glycan standards. The fetuin glycan sample showed peaks eluting at the corresponding positions of the glycan standards A1, A2, A3, and MAN-5. As expected from the literature (29,31), no peaks were seen corresponding to MAN-9, NA2, or NA3 (Figure 3). Several peaks were observed in the chromatograms obtained from analysis of glycan standards MAN-5, A1, A2, and A3. The A1, A2, and A3 glycan standards are isomeric glycans (Figure 1) and, thus, several peaks in the chromatograms from these glycans (Figure 3) could be due to separation of isomers. Altogether, the data from method A lead to the conclusion that MAN-9, NA2, and NA3 were absent while no conclusions could be made concerning identification of other oligosaccharides.

The results from HILIC method B showed peaks (Figure 4) eluting at the corresponding positions of the glycan standards A1, A2, A3, NA2, NA3, and MAN-9 in the fetuin glycan sample. No MAN-5 was detected in the fetuin sample as expected. HILIC method B, where 25mM ammonium acetate was used in the mobile phases, had an increased selectivity and resolution (Figure 4), compared to the method A (Figure 3). Because of the improved resolution, additional peaks appeared in the chromatograms of standards A1, A2, and A3, and the number of peaks in the mixture of the seven glycan standards and the glycan sample of fetuin was approximately doubled (Figure 4). The increased resolution and different selectivity made it possible, when combined with HILIC method A, to identify almost all

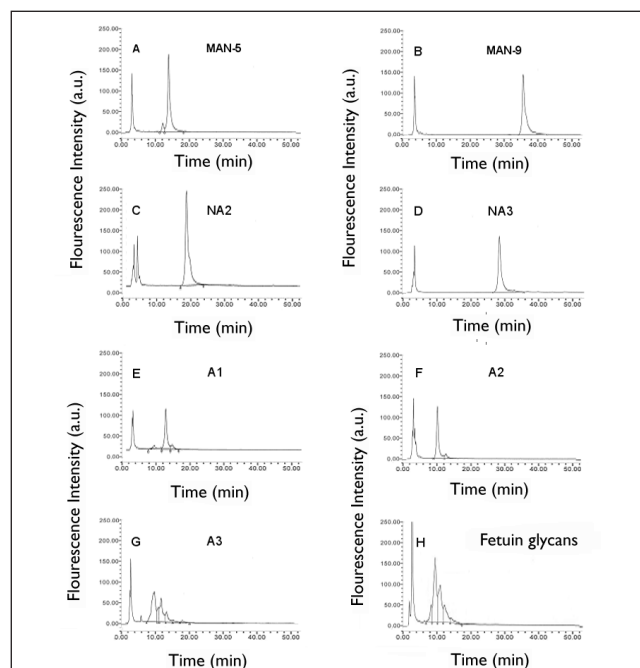


Figure 3. Analysis of 2-AB-labeled glycans by HILIC method A. The mobile phase used for gradient elution consisted of acetonitrile and 5mM ammonium acetate in water. MAN-5 (A), MAN-9 (B), NA2 (C), NA3 (D), A1 (E), A2 (F), A3 (G), and glycans from the fetuin sample (H).

peaks in the glycan sample of fetuin. For example, MAN-9 and A3 coeluted in method B. However, the use of method A showed that no MAN-9 was present in the glycan sample of fetuin, and this peak was therefore identified as glycan A3. With the same approach, we concluded that neither NA2 nor NA3 was present in fetuin glycans because their resolution from A3 was much better with HILIC method A. Peaks less than 0.5% of the total integrated area were not evaluated because these results were considered to be less reliable. Thus, the peaks that eluted at 13 and 19 min, when analyzing the fetuin glycans, were ignored (Figure 4).

To summarize the results, by combining HILIC method A and HILIC method B, the fetuin glycan sample was shown to contain glycans A1, A2, and A3, but no MAN-5 or MAN-9, which agrees with the literature (29,31). In addition, neither NA2 nor NA3 was found in this sample, which is also in accordance with previous reports, where these desialylated forms were shown to be absent, or present in very low concentrations (29,31). The final interpretation of the HILIC analyses of the fetuin glycan sample is shown in Figure 4H and Table I.

Discussion

The results of the relative glycan content of fetuin from this study are roughly in concordance with values found by Green et al. (29), although some discrepancy occurs (Table I). Townsend (31) et al. reported that the trisialylated glycan species of bovine

fetuin, obtained from Sigma, accounts for 68%, while Green et al. (29) reported a value of 54% (all having the A3 structure) for the same glycan species. The variations in the reported compositions of fetuin glycans may be explained by the differences in procedures for purifying fetuin and fetuin glycans, and also by the analytical methods. In addition, labeling procedures may contribute to discrepancies in the results. Green et al. (29) used techniques that included labeling with tritium [^3H], fractionation by IEX, and lectin affinity chromatography, followed by [^1H] nuclear magnetic resonance spectroscopy (NMR) analysis, while Townsend et al. (31) employed HPAEC–PAD. Several other techniques, including fluorescent labeling, NPLC, and MS (7,32) have also been frequently used for analyzing fetuin glycans. A few peaks in the fetuin glycan sample could not be identified, based on their retention times. These peaks probably correspond to oligosaccharides found in fetuin that differ from the purified glycan standards used in this work. The two HILIC methods presented should be evaluated in comparison with a reference method, preferably HPAEC–PAD, perhaps in combination with MS or NMR, to investigate the discrepancy of obtained results, compared to Green et al. (29), and also other reports should be considered. This should include characterization of unidentified peaks in the fetuin glycan sample and multiple peaks from some of the glycan standards.

An increased ammonium acetate concentration of 25mM increased the retention times for glycans A1, A2, and A3 (Figure 4), which have structures that bear one, two, and three sialic acid residues, respectively (Figure 1). In a previous work, we have shown that retention time increases for sialic acid and glucuronic acid, but not for uncharged monosaccharides when the ammonium acetate (pH 5.5) concentration is increased in the HILIC system using an Amide-80 column in an acetonitrile–water mobile phase (14). This may be explained by the increased formation of a hydrophilic complex, involving the carboxylic groups in the oligosaccharides and the ammonium ions in the mobile phase, which is more strongly retained, or that ammonia ions are bound to the HILIC column, to increase the electrostatic interaction with sialic acid residues of the glycans. The latter explanation would be comparable to ion pair chromatography (IPC), where the increased concentration of an ion-pair reagent in the mobile phases results in an increased retention, up to the concentration that saturates the Amide-80 column by the increased ion exchange effect (33). As the retention time in the chromatograms for the analyzed glycans tends to increase with an increasing number of sialic acid residues, we speculate that the unidentified glycans in the fetuin sample that elute in the end of the chromatogram (at about 42–50 min) might contain glycans bearing four or five sialic acids (Figure 4). The elution order of glycans A1 and A2 was reversed when using 25mM ammonium acetate (Figure 4) in the gradient, in comparison to 5mM (Figure 3).

N-Linked oligosaccharides have previously been analyzed using a range of different HPLC techniques, of which HPAEC–PAD is widely considered to be superior. Although HPAEC–PAD has a very high resolution, HILIC could be an important complement in analyzing oligosaccharides. The different resolution and selectivity obtained with different HPLC techniques are useful in combination to analyze and charac-

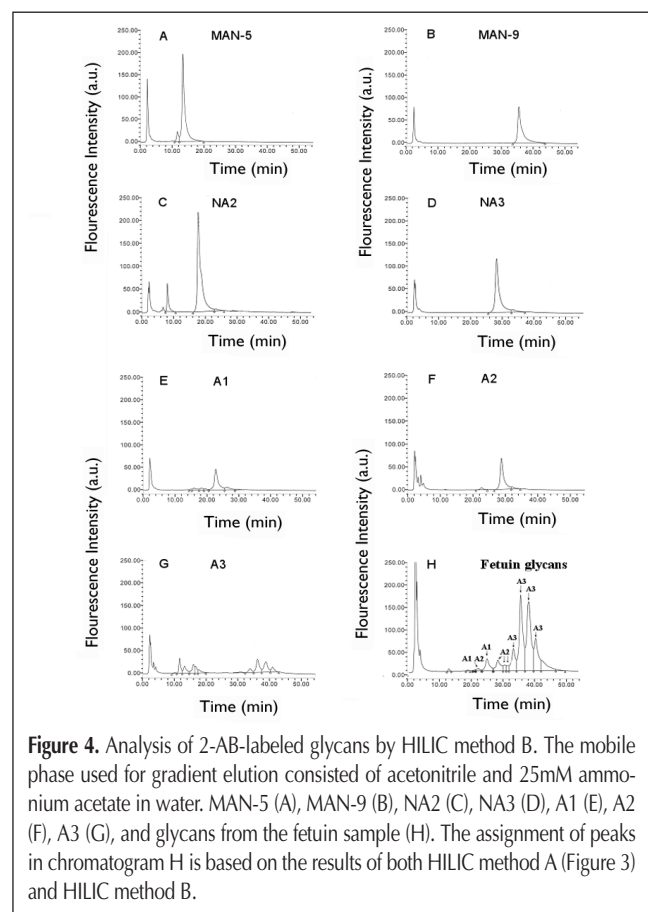


Figure 4. Analysis of 2-AB-labeled glycans by HILIC method B. The mobile phase used for gradient elution consisted of acetonitrile and 25mM ammonium acetate in water. MAN-5 (A), MAN-9 (B), NA2 (C), NA3 (D), A1 (E), A2 (F), A3 (G), and glycans from the fetuin sample (H). The assignment of peaks in chromatogram H is based on the results of both HILIC method A (Figure 3) and HILIC method B.

terize mixtures of isomeric glycans. Furthermore, HPAEC-PAD is associated with problems such as unstable baselines, noise, and the usage of high pH and high salt in the mobile phases (34), so that other chromatographic techniques may be more advantageous in analyzing glycans. HILIC, in contrast to HPAEC-PAD, can easily be combined with MS, due to the volatility of the mobile phases used in HILIC. However, by using ion suppression devices, HPAEC could be used with on-line MS detection (35), though the method would become more complicated in comparison with HILIC.

In several published works that describe the HILIC separation of carbohydrates, including oligosaccharides, acetonitrile has been used as an organic solvent in combination with water and various concentrations of ammonium acetate or ammonium formate buffers [ranging from no buffer at all (16), to 250mM ammonium formate (17–20)]. Separations of glycans have also been reported that use a binary gradient, with simultaneously increasing concentrations of ammonium formate and water for elution (20). The present work shows that the separation of compounds that differ in the number of carboxyl groups is strongly influenced by the buffer concentration (ammonium acetate), and thus can be used for optimization of HILIC analysis of *N*-linked complex glycans, and possibly other anionic substances as well. The two HILIC methods (A and B) presented here can be readily used in combination during the same analysis occasion, by using one concentrated ammonium acetate solution in the HPLC system, to provide a glycan profile that is highly characteristic of the analyzed glycoprotein. The combination method presented in this work may be applied to comparisons of glycan profiles of glycoproteins found in normal and disease states, or to characterize recombinant protein products.

In conclusion, the combination of two HILIC methods was shown to markedly facilitate the identification of complex mixtures of *N*-linked oligosaccharides and to provide high-resolution glycan profiles.

References

- E.I. Park, Y. Mi, C. Unverzagt, H.J. Gabius, and J.U. Baenziger. The asialoglycoprotein receptor clears glycoconjugates terminating with sialic acid- α 2,6GalNAc. *Proc. Natl. Acad. Sci. U.S.A.* **102**: 17125–17129 (2005).
- H. Nakagawa, Y. Kawamura, K. Kato, I. Shimada, Y. Arata, and N. Takahashi. Identification of neutral and sialyl *N*-linked oligosaccharide structures from human serum glycoproteins using three kinds of high-performance liquid chromatography. *Anal. Biochem.* **226**: 130–138 (1995).
- N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata, and N. Takahashi. Analyses of *N*-linked oligosaccharides using a two-dimensional mapping technique. *Anal. Biochem.* **171**: 73–90 (1988).
- J.P. Liu, O. Shirota, D. Wiesler, and M. Novotny. Ultrasensitive fluorometric detection of carbohydrates as derivatives in mixtures separated by capillary electrophoresis. *Proc. Natl. Acad. Sci. U.S.A.* **88**: 2302–2306 (1991).
- L.Y. Frado and J.E. Strickler. Structural characterization of oligosaccharides in recombinant soluble human interferon receptor 2 using fluorophore-assisted carbohydrate electrophoresis. *Electrophoresis.* **21**: 2296–2308 (2000).
- M.R. Hardy and R.R. Townsend. Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection. *Proc. Natl. Acad. Sci. U.S.A.* **85**: 3289–3293 (1988).
- K.R. Anumula and S.T. Dhume. High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. *Glycobiology* **8**: 685–694 (1998).
- Y.C. Lee, B.I. Lee, N. Tomiya, and N. Takahashi. Parameterization of contribution of sugar units to elution volumes in reverse-phase HPLC of 2-pyridylaminated oligosaccharides. *Anal. Biochem.* **188**: 259–266 (1990).
- K.R. Anumula. Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. *Anal. Biochem.* **350**: 1–23 (2006).
- M.L. Bergh, P. Koppen, and D.H. van den Eijnden. High-pressure liquid chromatography of sialic acid-containing oligosaccharides. *Carbohydr. Res.* **94**: 225–229 (1981).
- A. Berthod, S.S.C. Chang, J.P.S. Kullman, and D.W. Armstrong. Practice and mechanism of HPLC oligosaccharide separation with a cyclodextrin bonded phase. *Talanta* **47**: 1001–1012 (1998).
- A.J. Alpert. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* **499**: 177–196 (1990).
- A.J. Alpert, M. Shukla, A.K. Shukla, L.R. Zieske, S.W. Yuen, M.A.J. Ferguson, A. Mehlert, M. Pauly, and R. Orlando. Hydrophilic-interaction chromatography of complex carbohydrates. *J. Chromatogr. A.* **676**: 191–202 (1994).
- G. Karlsson, S. Winge, and H. Sandberg. Separation of monosaccharides by hydrophilic interaction chromatography with evaporative light scattering detection. *J. Chromatogr. A.* **1092**: 246–249 (2005).
- G. Karlsson, A.C. Hinz, and S. Winge. Determination of the stabilizer sucrose in a plasma-derived antithrombin process solution by hydrophilic interaction chromatography with evaporative light scattering detection. *J. Chromatogr. Sci.* **42**: 361–365 (2004).
- H. Kakita, H. Kamishima, K. Komiya, and Y. Kato. Simultaneous analysis of monosaccharides and oligosaccharides by high-performance liquid chromatography with postcolumn fluorescence derivatization. *J. Chromatogr. A.* **961**: 77–82 (2002).
- Y. Takegawa, K. Deguchi, T. Keira, H. Ito, H. Nakagawa, and S. Nishimura. Separation of isomeric 2-aminopyridine derivatized *N*-glycans and *N*-glycopeptides of human serum immunoglobulin G by using a zwitterionic type of hydrophilic-interaction chromatography. *J. Chromatogr. A.* **1113**: 177–181 (2006).
- V.V. Tolstikov and O. Fiehn. Analysis of highly polar compounds of plant origin: Combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. *Anal. Biochem.* **301**: 298–307 (2002).
- G.W. Byrne, A. Schwarz, J.R. Fesi, P. Birch, A. Nepomich, I. Bakaj, M.A. Velardo, C. Jiang, A. Manzi, H. Dintzis, L.E. Diamond, and J.S. Logan. Evaluation of different alpha-Galactosyl glycoconjugates for use in xenotransplantation. *Bioconjug. Chem.* **13**: 571–581 (2002).
- G.R. Guile, P.M. Rudd, D.R. Wing, S.B. Prime, and R.A. Dwek. A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. *Anal. Biochem.* **240**: 210–226 (1996).
- N*-glycanase (Peptide-*N*-Glycosidase F), Prod. No. GKE-5006. Product information from ProZyme/Glyko, San Leandro, CA, USA.
- K.P. Keinänen. Effect of deglycosylation on the structure and hormone-binding activity of the lutropin receptor. *Biochem J.* **256**: 719–724 (1988).
- Phast System. Separation Technique File No. 111. SDS-PAGE in homogeneous media. Pharmacia LKB Biotechnology AB, Uppsala, Sweden.
- Phast System. Technical Note No. 2. Two-dimensional electrophoresis with PhastGel separation media. Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

25. GlycoClean R Cartridges, Prod. No. GKI-4756. Product information from ProZyme/Glyko, San Leandro, CA.
26. Signal 2-AB Labelling Kit, Prod. No. GKK-404. Product information from ProZyme/Glyko, San Leandro, CA.
27. GlycoClean S Cartridges, Prod. No. GKI-4726. Product information from ProZyme/Glyko, San Leandro, CA.
28. J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, and R.B. Parekh. Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Anal. Biochem.* **230**: 229–238 (1995).
29. E.D. Green, G. Adelt, J.U. Baenziger, S. Wilson, and H. Van Halbeek. The asparagine-linked oligosaccharides on bovine fetuin. Structural analysis of *N*-glycanase-released oligosaccharides by 500-megahertz ¹H NMR spectroscopy. *J. Biol. Chem.* **263**: 18253–18268 (1988).
30. Y.Q. Yu, M. Gilar, J. Kaska, and J.C. Gebler. Deglycosylation and Sample Clean-up Method for Mass Spectrometry Analysis of *N*-linked Glycans. The Applications Book. Waters Corporation, Milford, MA, 2005, pp. 34–36, 2005.
31. R.R. Townsend, M.R. Hardy, D.A. Cumming, J.P. Carver, and B. Bendiak. Separation of branched sialylated oligosaccharides using high-pH anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* **182**: 1–8 (1989).
32. B. Kuster, S.F. Wheeler, A.P. Hunter, R.A. Dwek, and D.J. Harvey. Sequencing of *N*-linked oligosaccharides directly from protein gels: in-gel deglycosylation followed by matrix-assisted laser desorption/ionization mass spectrometry and normal-phase high-performance liquid chromatography. *Anal. Biochem.* **250**: 82–101 (1997).
33. L.R. Snyder, J.L. Glajch, and J. Kirkland. Practical HPLC Method Development, 2nd ed. Wiley Interscience, New York, NY, 1997, p. 320.
34. K.R. Anumula and P.B. Taylor. Rapid characterization of asparagine-linked oligosaccharides isolated from glycoproteins using a carbohydrate analyzer. *Eur. J. Biochem.* **195**: 269–280 (1991).
35. H.A. Schols, M. Mutter, A.G. Voragen, W.M.A. Niessen, R.A van der Hoeven, J. van der Greef, and C. Bruggink. The use of combined high-performance anion-exchange chromatography-thermospray mass spectrometry in the structural analysis of pectic oligosaccharides. *Carbohydr. Res.* **261**: 335–342 (1994).

Manuscript received December 2, 2006;
revision received June 6, 2007.