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Isolation and Purification of Glycoconjugates from Complex Biological Sources by Recycling HPLC

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Abstract

Among of the most urgent needs of the glycobiology community is to generate libraries of pure carbohydrate standards. While many oligosaccharides have recently been synthesized, some glycans of biomedical importance are still missing in existing collections, or are available in only limited amounts. To address this need, we demonstrate the use of the relatively unexplored technique of recycling high-performance liquid chromatography (R-HPLC) to isolate and purify glycoconjugates from several natural sources. We were able to routinely achieve purities greater than 98%. In several cases, we were able to obtain isomerically pure substances, particularly for glycans with different positional isomerism. These purified substances can then be used in different analytical applications, for example, as standards for mass spectrometry (MS) and capillary-based separations. Moreover, using a bifunctional aromatic amine, the same derivatization agent can be used to enable UV detection of oligosaccharides during their purification and link the isolated molecules to functionalized surfaces and potentially create glycan arrays.

Keywords

recycling HPLC; hydrophilic-interaction chromatography; glycans; carbohydrates; glycan arrays; 4-(2-aminoethyl)aniline

Introduction

Important biological roles of glycoconjugates and their interactions with other biomolecules (sugar-protein and sugar-sugar interactions) have now been widely recognized through the rapidly growing literature on complex carbohydrates. Arguably, the greatest impact of this knowledge may be in biomedical investigations, where understanding glycobiology is pertinent to the fields of immunology, disease biomarker discovery, the design of modern biologic pharmaceuticals, and the goals of personalized medicine.¹⁻⁴ In addition to the health-related areas, a recent report by the National Research Council⁵ also notes the importance of glycoscience to biomaterials and energy research, emphasizing its broad potential impact on our society.

Numerous recent achievements in glycobiology have been aided significantly by analytical advances and instrumentation, particularly biomolecular mass spectrometry (MS) and various separation techniques, which together continue to contribute to the documentation of new biologically significant glycans.^{6,7} Additionally, the concerted efforts in oligosaccharide synthesis⁷ are rapidly generating an ever-increasing collection of carbohydrates and a joint collaborative effort of experts in these diverse fields has led to the

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concept of and realization of glycan arrays.⁸ This area of research is still under development and refinement. While many of the glycans that have been incorporated into the current arrays have been synthetic products,⁹ many biologically relevant structures remain unavailable.

To complement the synthetic and biosynthetic efforts in other laboratories, this communication describes the isolation of pure natural glycans from several complex biological sources, including common glycoproteins and human breast milk, through the use of recycling HPLC (R-HPLC),¹⁰ a powerful but long-neglected approach. We demonstrate that the glycans of interest can be obtained at very high levels of purity. The method of recycling chromatography performed in this study utilized twin columns located on the opposite sides of a switching valve. This procedure allows the control of optimized column lengths, or the number of “effective columns” that analytes experience by redirecting the effluent of one column to the inlet of the other (see Figure 1, below). As a direct result, the overall resolution of the system is also controlled in a reproducible and predictable manner, and given a sufficient number of column lengths, closely related structures (such as isomeric species) can be resolved from each another. It should be noted that this recycling approach is fundamentally different from a commonly-used single-column method where the column effluent is circulated back through the LC pump and reintroduced into the same column.

In one of the early applications of the “twin column” method, described in 1998, Lan and Jorgenson were able to baseline-resolve phenylalanine and D⁵-phenylalanine, somewhat surprisingly, in just 90 minutes.¹⁰ Interestingly, this method has not since been applied to complex samples to isolate/purify compounds of potential biochemical interest. We reasoned that such a technique would be capable of resolving a number of carbohydrates, including different isomeric species, and would thus result in the recovery of analytes of sufficient purity needed for a number of analytical applications. Beyond incorporating structurally diverse glycoconjugates molecules in the most up-to-date glycan arrays, highly purified analytes can also be used for structural verification (as needed with different isomeric variants in glycomic investigations) in MS and tandem MS studies. Further potential applications of highly purified carbohydrates include their use as retention/migration time standards for LC and capillary electrophoresis (CE), thus permitting confident structural assignments. There are other potential applications and measurements (binding studies, development of clinical reagents, etc.) in glycoscience which would also benefit from having a large library of readily available, highly purified glycans.

Experimental

Materials

Amide-80 HPLC columns (4.6 × 250 mm, 5-μm particle size) and guard columns (3.2 × 15 mm, 5-μm particle size) were acquired from Tosoh Bioscience, LLC (King of Prussia, PA). EMD Chemicals, Inc. (Gibbstown, NJ) was the source for HPLC-grade water and the HPLC-grade acetonitrile (ACN), while dimethylsulfoxide (DMSO) and acetic acid were acquired from Mallinckrodt Baker (Phillipsburg, NJ). Sigma Chemical Co. (St. Louis, MO) provided 2',4',6'-trihydroxyacetophenone monohydrate (THAP), ammonium acetate, β-mercaptoethanol (βME), 4-aminobenzamide (4-AB), sodium cyanoborohydride, 4-(2-aminoethyl)aniline, ribonuclease B, α₁-acid glycoprotein (AGP), and bovine fetuin. N-Glycanase F (PNGase F) was purchased from Northstar Bioproducts (East Falmouth, MA). The graphite spin columns used for sample purification were obtained from Harvard Apparatus (Holliston, MA) and 2,5-dihydroxybenzoic acid (2,5-DHB) was received from Alfa Aesar (Ward Hill, MA). Sodium dodecylsulfate (SDS) was acquired from Bio-Rad Laboratories (Hercules, CA), while Nonidet P-40 was purchased from Roche Diagnostics (Indianapolis, IN). Chloroform and methanol were received from Macron (Center Valley,

PA) and N-hydroxysuccinimide-functionalized glass slides were products of Microsurfaces, Inc. (Englewood, NJ). The fluorescently-labeled lectins, *Sambucus nigra* agglutinin (SNA) and *Aleuria Aurantia* lectin (AAL), were acquired from Vector Laboratories (Burlingame, CA), while human breast milk was supplied by Bioreclamation, LLC (Westbury, NY).

Carbohydrate Isolation

Glycans derived from glycoprotein standards were released using PNGase F. Briefly, 1-5 mg of a glycoprotein standard were solubilized in 500 μ L of a buffer composed of 10 mM sodium phosphate (pH = 7.5)/0.1% SDS/0.1% β ME and incubated at 60° C for 1 h. After allowing the samples to cool to room temperature, 50 μ L of a 10% (v/v) solution of Nonidet P-40, resulting in a final concentration of 0.1%, were added and allowed to equilibrate for 5 min. Next, a 2- μ L aliquot containing 5 mU of PNGase F was added and the samples were incubated at 37° C for 18 h. Following the digestion, proteins were removed by precipitation through adding 2.5 mL of a 2:1 (v/v) chloroform/methanol solution. After gently shaking, the samples were centrifuged, the upper layer containing the glycans was removed, and dried using a vacuum centrifuge. The glycans were further purified using graphite spin columns and then dried.

Similarly, free carbohydrates from 100- μ L aliquots of human milk were isolated by first precipitating proteins with 500 μ L of the 2:1 (v/v) chloroform:methanol solution. After gently shaking, centrifuging, removing the upper layer, and drying, the oligosaccharides were purified using graphite spin columns and dried.

Reducing-end Modification for UV Detection

To facilitate UV detection during the isolation/purification by HPLC, the reducing ends of some carbohydrates were derivatized with either 4-aminobenzamide or 4-(2-aminoethyl)aniline via reductive amination. To perform these reactions, 100 μ L of a 70%/30% solution of a DMSO/acetic acid solution was prepared and 6 mg of 4-AB or 10 μ L of 4-(2-aminoethyl)aniline were added along with 6 mg of sodium cyanoborohydride. A 25- μ L aliquot of this labeling solution was added to the carbohydrate sample and incubated for ca. 15 h at 37° C. Subsequently, the samples were purified using a hydrophilic-interaction chromatography (HILIC) (Amide-80) medium loaded into microspin centrifuge columns. For this clean-up procedure, the medium was washed with 400 μ L of a solution composed of 95%/5%/0.1% water/ACN/trifluoroacetic acid (TFA) three times, followed by 400 μ L of a 85%/15%/0.1% ACN/water/TFA solution three times. The sample solution was adjusted to a volume of 400 μ L with a composition of 85%/0.1% ACN/trifluoroacetic acid and applied to the column and centrifuged. The eluent was then reapplied an additional two times. After washing the medium twice with 250- μ L aliquots of the 85%/15%/0.1% ACN/water/TFA solution, the glycans were eluted with two 200- μ L aliquots of the 95%/5%/0.1% water/ACN/TFA solution. Following their elution, the samples were dried in a vacuum centrifuge.

Carbohydrate Purifications by R-HPLC

This study used a Dionex P680 HPLC instrument, equipped with an autosampler and a PDA-100 UV detector monitoring wavelengths of 298 nm (for 4-AB-derivatized sugars) or 250 nm (for 4-(2-aminoethyl)aniline-modified carbohydrates). At the heart of this chromatographic system were two identical Amide-80 columns (4.6 $\times$ 250 mm, packed with 5 μ m particles) located on opposite sides of a switching valve (see Figure 1 for the valve configuration). The system was operated in a HILIC-type mode of chromatography at a flow rate of 0.9 mL/min (unless otherwise indicated) and the separations were performed at 60° C (unless otherwise noted). In this study, mobile phase A was an aqueous 10 mM ammonium acetate buffer (pH = 7.0) and mobile phase B was acetonitrile. Under isocratic elution conditions, 60% mobile phase B was applied for oligosaccharides labeled with 4-AB. We

noticed that sugars labeled with the aniline derivative were exhibited somewhat longer retention times through a single column using the mobile-phase conditions for 4-AB-labelled carbohydrates, which resulted in excessively long recycling experiments. To balance the recovery of high-purity solutes with a reasonable purification time, we performed recycling experiments using 50% mobile phase B for oligosaccharides tagged with 4-(2-aminoethyl)aniline.

For carbohydrates that required more extensive recycling, the UV trace was monitored to determine the retention time, $t_{\min}$, of an analyte of interest through a single column. The valve was then actuated at $1.5t_{\min}$, i.e., the time when the analyte had migrated halfway through the second column, which redirected the column effluent back to the inlet of the first column. The valve was then actuated at a time of $1.5t + t_{\min}$, which corresponded to the time when the analyte had returned to the midpoint of the first column. This process was repeated as many times as deemed necessary. When appropriately pure, the carbohydrates of interest were collected, dried, and analyzed by matrix-assisted laser desorption/ionization (MALDI) tandem time-of-flight (TOF) mass spectrometry (MS).

Mass Spectrometry

The collected peaks were further analyzed by an Applied Biosystems (Foster City, CA) 4800 MALDI tandem TOF MS for detailed structural characterizations. Neutral structures were analyzed in the instrument's positive-ion mode, using 2,5-DHB (10 mg/mL in 50%/50% water/methanol, supplemented with 1 mM sodium acetate and dried under vacuum) as the matrix. Positive-mode MS experiments used 1,000 laser shots, while 2,000 laser shots were collected for tandem MS experiments. For sialylated structures, which bear a negative charge, THAP (5 mg/mL in 50%/50% water/ACN, supplemented with 2.5 mg/mL ammonium citrate and dried under vacuum) was used as the matrix. Negative-mode experiments were also performed with 1,000 laser shots. The MS methods reported here fulfill the Minimum Information Required for a Glycomics Experiment (MIRAGE) (http://glycomics.ccruc.uga.edu/MIRAGE/index.php/MS_guideline) guidelines.

Attachment of Purified Glycans to Glass Surfaces

Following their purification, selected glycans which had been derivatized with 4-(2-aminoethyl)aniline were attached to glass surfaces functionalized with N-hydroxysuccinimide. Surface attachment occurs through the aliphatic amine of the tagging chromophore. To do this, glycans were resuspended in a 50 mM sodium phosphate coupling buffer (pH = 8.0). A 0.25- μ L aliquot was spotted on the slide, along with 0.25 μ L of a control solution composed of the coupling buffer containing 100 mM TRIS (pH = 8.0). After allowing the spots to dry at room temperature, the slides were washed twice with 1-mL aliquots of the coupling buffer, and 5 $\times$ with 1-mL aliquots of HPLC-grade water. An appropriate fluorescently-labeled lectin, diluted to a suitable working concentration in a binding buffer (50 mM sodium phosphate with 200 mM sodium chloride at pH = 7.5, was added to the slide and allowed to bind for 20 min, after which time the slide was washed twice with 1-mL aliquots of the coupling buffer and 5 $\times$ 1-mL aliquots of HPLC-grade water. The spots were then visualized by fluorescence microscopy to confirm lectin binding to the immobilized carbohydrates.

Results and Discussion

General Goals

In 1998 Lan and Jorgenson¹⁰ demonstrated that R-HPLC could be used effectively to resolve structurally-close solutes in the twin-column format. Their specific application involved aromatic amino acids with a native UV chromophore. In order to establish R-

HPLC as a preparative separation methodology for glycoscience applications, we needed to investigate first the applicability of this approach to structurally similar carbohydrates and second, the UV-tagging options for glycans in the microgram-to-milligram range, to be compatible with the reasonable amounts of glycoproteins and other biomaterials. Our overall objective has been to isolate structurally close glycoconjugates in their pure state as analytical standards.

In order to advance our first objective, we studied artificial mixtures of non-reducing sugars (detected through UV at 185 nm) during the HILIC mode of separation. As shown in Figure 2, with a model mixture of equimolar amounts ($\sim 600 \mu\text{g}$ each) the trisaccharides raffinose (a linear structure) and melezitose (a branched trisaccharide), after 4 recycling HILIC steps (an effective column length of 1.00 m) at room temperature and a flow rate of 0.4 mL/min, only a single chromatographic peak is detected. However, the mixture components soon began splitting into two peaks, achieving a respectable resolution after 10 recycling operations, or 2.50 m of total column length. It is important to note that even at approximately 600 μg of sample loaded for each oligosaccharide, peaks with acceptable symmetry were still observed. We initially observed what appeared to be a decrease in the peak height during the first several recycling steps, consistent with the previous report for this technique.¹⁰ Correspondingly, there was an increase in the peak width, preserving a constant peak area. Eventually, the peak widths seemingly became stabilized, as they did in the initial report,¹⁰ which was attributed to the pressure gradient across the concentration profile. Importantly, the integrated peak areas throughout the separation remained very constant, indicating that no sample losses were occurring.

Many glycans and carbohydrates of interest to us have a reducing end in their structures so that a suitable chromophore can be incorporated into the oligosaccharides to enhance their UV detection. We can thus more effectively monitor the purifications of glycans derived from typical glycoproteins and biological samples. Initially, we demonstrated the effectiveness of recycling tagged carbohydrates with another model mixture with subtle differences in their structures, the linear trisaccharides maltotriose and cellotriose, which differ in their α and β anomeric linkages. For this analysis, conducted at 40° C and a flow rate of 0.6 mL/min, 50 μg of each carbohydrate were injected, appearing initially as a single peak. After three effective column lengths, two separate peaks began to develop and the analytes became fully resolved as their 4-aminobenzamide derivatives after 29 recycling passes (an effective column length of 3.5 meters) and detected with an adequate sensitivity at a wavelength of 298 nm (see Figure 3). This experiment further highlighted that R-HPLC can separate carbohydrates with minor differences in their structures in a reasonable time (less than 3 h). (Note that the initial recycling experiments used a slightly different valve configuration that allowed analytes to be detected after each column. However, due to higher back pressures, we reconfigured the valving set-up shown in Figure 1 in order to maintain a safe operating pressure for the detector cell used in this work.)

Our laboratory is focused on the characterization of glycans whose structures are altered due to pathological conditions.^{11,12} We have previously shown that in addition to the changes in abundance levels, the ratios of different isomeric species are also altered in different cancers. This has been demonstrated for core vs. outer-arm fucosylation^{11,12} and for the different linkages of sialic acids.^{12,13} With some structural uncertainties remaining, it is important to obtain isomerically pure carbohydrates for more detailed studies, such as possibly including them in a glycan array for diagnostically important protein-binding situations. Our first investigation into resolving positional isomers utilized another model system, a 4-AB-labeled N-glycan mixture derived from bovine fetuin. This separation is illustrated in Figure 4, demonstrating the isolation and a further recycling of a triantennary/tetrasialylated glycan. A relatively minor peak, labeled as Peak 1, was selected initially as the parent structure,

developing shoulders through recycling into three discrete fractions (after using an effective column length of 30 meters and a total recycling time of nearly 30 h). These three peaks corresponded to the three different possible locations of the fourth sialic acid.

Isolating Positional Isomers of High-mannose Glycans

Protein glycosylation is extremely important in the processes related to the interactions between the virus envelope proteins and the host receptor proteins during the virus entry. Among the N-glycans that appear to be the key structures regulating the success of virus entry^{14,15} are the high-mannose oligosaccharides. In order to facilitate different investigations of the host-pathogen interactions and studies aiming ultimately at the development of successful vaccines, it would be desirable to develop glycan arrays of the general type explored previously for the benefit of early cancer diagnostic and prognostic evaluations.^{16,17} While many synthetically prepared glycans have now become available for the attachment to the surfaces of arrays,^{16,17} the availability of high-mannose structures, and particularly those which are isomerically pure, has remained limited. Natural sources such as ribonuclease B¹⁸ or yeast external invertase¹⁹ can provide an alternative. Due to the propensity of some of these glycans, particularly Man-7 and Man-8, to form different positional isomers, their isolation is not a trivial task. Supplementary Figure 1 depicts an LC trace for the high-mannose glycans associated with bovine ribonuclease B. The merits of R-HPLC, in this regard, are demonstrated in Figure 5A-D, which shows resolution of the three possible isomers associated with the Man-7 parent glycan. After selecting this parent structure for purification, the D2 isomer (see structures in Figure 5), began to be well resolved from the D1 and D3 isomers after three effective columns (recycled once) (Figure 5A). The D1 and D3 isomers began to show signs of resolution after 15 effective columns (Figure 5B), and were baseline-resolved after 35 effective columns (Figure 5C). Additional recycling did not seem to significantly improve the separation (Figure 5D). Thus, after 35 effective columns, these two structures were sufficiently resolved for collection at a purity suitable for further applications. Following their collection, the structure of each isomer was confirmed through tandem MS fragmentation and NMR analyses (results not shown).

Human Milk Oligosaccharide Isolations

Not only is human milk a protein-rich fluid containing several glycoproteins featuring extensive O-linked glycosylation, but it is also an abundant source of free carbohydrates. While lactose is the major carbohydrate constituent, up to 200 different structures (including various monosaccharide sequences, linkages and positions, and the presence of charged groups) are encountered at different abundances,^{20,21} with an estimated total carbohydrate concentration to be 5-15 mg/mL. Human milk oligosaccharides have been of considerable interest in relation to the management of the human microbiome, interactions within the infant intestinal epithelium, and even possibly modulation of the immune system.²² Human milk represents one of the most wide-ranging and abundant sources of free oligosaccharides, many of which are unique to humans. Since such oligosaccharides may act as more or less potent adhesion ligand analogs and immune modulators, it appears attractive to place pure isolates from this abundant natural source on glycan arrays.

To demonstrate further the ability of R-HPLC in the isolation of high-purity milk oligosaccharides, we briefly characterized the free carbohydrates isolated from a pooled milk sample, as shown in the MALDI-MS profile (Figure 6A). Figure 6B shows the recycling purification of selected human milk oligosaccharides. In this purification, a peak with a longer retention time, ca. 7.5 minutes, was selected for further analysis (refer to Figure 6C). While this chromatographic feature was initially present as only a single peak, it began to split into two peaks after being subjected to 5 effective columns (column length of 1.25 m). The resolution between the two peaks, referred to as Peak A and Peak B, continued

to increase with each subsequent recycling pass. After the 9th effective column (2.25 m column length), the peaks were essentially baseline-resolved (see Figure 6D) and deemed sufficiently pure to be collected and subjected to MALDI MS analysis. To determine the chemical nature of the analytes, MALDI TOF-MS was employed. This analysis revealed that several ions were present below the m/z value of ca. 800 due to the matrix cluster ions, that is, they were not chemical entities present in the purified sample. However, a compound giving rise to an ion detected at an m/z value of 1142.3 was present in each collected chromatographic peak, as shown in Figures 6E and G, for Peaks A and B, respectively. These ions correspond to the doubly-fucosylated isomeric hexasaccharides, LNDFH I and II. To more definitively assign the structures for each chromatographic peak, tandem MS was employed. As depicted in Figure 6F, the tandem MS spectrum for the analyte present in Peak A, a fragment ion at an m/z value of 631.0 was present, which was attributed to the fragment Gluc(Fuc)-Gal, which is only present for LNDFH II. Similarly, the analyte present in Peak B produced fragment ions represented by the m/z values of 698.0, which was the Gal(Fuc)-GlcNAc(Fuc) fragment, 834.1, corresponding to the GlcNAc(Fuc)-Gal-Gluc fragment, and 832.1, which indicated the presence of a Gal(Fuc)-GlcNAc(Fuc)-Gal fragment. These ions could only originate from LNDFH I.

Based on the MS data, the estimated purity for each isomer was greater than 99%. At this level of purity, the isolated analytes could function as analytical standards and array constituents for probing important protein-carbohydrate interactions. While this example indicates a potential application of R-HPLC in the area involving the location of one of the fucosyl substitutions in a hexasaccharide structure, we have further demonstrated the ability of this method to resolve core vs. outer-arm fucosylation in somewhat smaller glycans present in other complex biological materials (data not shown).

Attachment of Purified Glycans to Glass Surfaces

One of the potential applications for oligosaccharides purified by this technology can be in the construction of new types of glycan arrays. Even though some of the devices currently being used have hundreds of different carbohydrates attached,^{16,17} many of these are small sugars of primarily synthetic origin. To gain a more detailed biochemical understanding of the glycans associated with different diseases, for example the binding partners for proteins over-expressing the sialyl Lewis^x epitope in cancer, both N- and O-linked glycans need to be included. Complementing the current efforts to synthesize these types of structures, various oligosaccharides can readily be purified from proteins for attachment to chip surfaces.

One of the more popular ways to attach a glycan to surface materials is to react an analyte amine with surface-immobilized N-hydroxysuccinimide (NHS). While amine groups are prevalent in other biomolecules, such as peptides and proteins, they must be introduced chemically to the carbohydrate structure. Thus, a suitable compound for creating glycan arrays with carbohydrates isolated and purified by R-HPLC should have two amino groups with different reactivities, one amine suitable for its attachment to the carbohydrate, and the second to react with the surface-attached NHS. An aromatic moiety for improving the UV detection during the LC purification is also highly desirable. Fortunately, 4-(2-aminoethyl)aniline, which is commercially available, is the reagent that meets all of these requirements. Importantly, this molecule has both an aromatic and aliphatic amine, which have significantly different pK_a values. This allows only the aromatic amine to be reactive under the reductive amination conditions, eliminating the possibility of crosslinking two carbohydrates through the aliphatic amine. Conversely, the attachment of a derivatized carbohydrate to the surface is performed at slightly basic conditions and occurs through the aliphatic amine. The attachment of derivatized glycans to NHS-activated surfaces is depicted in Supplementary Scheme 1.

To demonstrate this scheme, we first subjected the glycans derived from human α_1 -acid glycoprotein (AGP) to a reductive amination procedure using 4-(2-aminoethyl)aniline as the tagging group. AGP is among the serum glycoproteins which function as acute-phase responders in a number of inflammatory conditions and cancer. Moreover, this protein is decorated with a number of tri- and tetra-antennary glycans, many of which are fucosylated. Consequently, the glycans of this protein are of significant interest for further glycomic biomedical studies.

As demonstrated in Figure 7a, two different glycans were obtained at a very high level of purity (greater than 98%) in an R-HPLC isolation that had a runtime of just over 3 h. These were both triantennary/trisialylated glycans, with the earlier-eluting peak containing the afucosylated analyte, as indicated by the MALDI MS shown in Figure 7b. The later-eluting peak contained the fucosylated analogue of this carbohydrate, whose structure was also confirmed by MALDI MS (see Figure 7c). Unfortunately, we were unable to pinpoint the exact location of the fucose moiety at this time as the tandem MS of this analyte was rather uninformative.

Following the conjugation of the aliphatic amine with the NHS-derivatized glass surfaces, lectins derivatized with fluorescein were allowed to bind to the immobilized carbohydrates. Since lectins are proteins that bind specific sugars, for example *Sambucus nigra* agglutinin (SNA) interacts with sialic acids while *Aleuria aurantia* lectin (AAL) will bind fucose, these reagents were used to detect and confirm the attachment of different carbohydrates. As shown in Figure 7d, fluorescence was detected using SNA for the afucosylated glycan and was similarly detected when AAL was introduced to the spots containing the fucosylated analogue (Figure 7e). Importantly, no fluorescence was observed for the control spots. While these are preliminary experiments, the results confirm the ability to couple highly purified carbohydrates from natural sources to a glass surface. Presently, we are actively working to incorporate these and other structures, purified by R-HPLC, onto glass slides using more modern printing technologies with promising results (data not shown).

Conclusions

We have demonstrated here the capabilities of recycling HPLC for the fractionation of complex glycoconjugate mixtures and the purification of glycan derivatives in the microgram-to-milligram range. The applications of this methodology in the glycoscience arena are likely to complement the synthetic efforts in providing high-purity carbohydrate standards through their isolation from natural sources. The feasibility of our approach has been demonstrated through the isolation of complex N-linked glycans from glycoproteins and fucosylated isomeric oligosaccharides from human breast milk. Through the use of 4-(2-aminoethyl)aniline to tag the N-glycans of human AGP and further attach them to a derivatized glass surface, we also have demonstrated the potential of this approach for the preparation of glycan arrays with structures that are currently difficult to synthesize. Isolation of natural high-purity glycoconjugates as standards for HPLC and CE measurements has also been indicated as potential applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of the valve and column configuration used for recycling HPLC used for most of the recycling experiments in this study.

Figure 2. RecyclingHPLC chromatogram demonstrating the resolution of the isomeric trisaccharides melezitose and raffinose (600 μg each). (A slightly different valve configuration was used in this experiment than is shown in Figure 1.)

Figure 3.

Recycling HPLC chromatogram demonstrating the power of the technique to separate linear trisaccharides differing only in their anomeric configurations (α vs. β linkages). (A slightly different valve configuration was used in this experiment than is shown in Figure 1.)

Figure 4. Recycling HPLC chromatogram demonstrating the separation of the triantennary/tetrasialylated isomeric glycans derived from bovine fetuin.

Figure 5. Recycling HPLC isolation and purification of the D1-3 isomers of the mannose₇ glycan derived from bovine ribonuclease B.

Figure 6.

A) MALDI MS profile of free oligosaccharides present in human breast milk. B) R-HPLC chromatogram for the isolation and purification of human milk oligosaccharides. C) a single chromatographic peak (highlighted) that D) split into two peaks (A and B) due to recycling. E) and G) MALDI MS analysis of the Peaks A and B, respectively. F) and H) tandem MS of Peaks A and B respectively to definitively determine the structure of each analyte.

Figure 7.

a) R-HPLC chromatogram of a triantennary-trisialylated glycan and its fucosylated analogue derived from human α_1 -acid glycoprotein; b) and c) negative-mode MALDI mass spectra for the triantennary-trisialylated glycan and its fucosylated version, respectively; d) and e) triantennary-trisialylated glycan and its fucosylated analogue, respectively, immobilized on a glass surface and stained with an appropriate fluorescently-labeled SNA or AAL, respectively.