Fractionation and Analysis of Neutral Oligosaccharides by HPLC

UNIT 17.21B

This unit describes the fractionation and analysis of neutral oligosaccharides by high-performance liquid chromatography (HPLC) on bonded amine columns. Separation is achieved with gradients of acetonitrile and water and is based upon hydrogen bonding between the NH_2 groups of the column and the hydroxyl groups of the oligosaccharides, as described in the Basic Protocol. The Support Protocol describes the reduction and desalting of neutral oligosaccharides with sodium borohydride. The basic principles of HPLC are discussed in UNITS 10.12-10.14 and in Snyder et al. (1988).

SEPARATION OF NEUTRAL OLIGOSACCHARIDES ON A BONDED AMINE HPLC COLUMN

BASIC PROTOCOL

Neutral oligosaccharides bind to amine-bonded HPLC columns because of hydrogen bonding via hydroxyl groups, which is promoted by acetonitrile and disrupted by water. The sample is loaded in a high concentration of acetonitrile and the column is developed with a gradient of increasing water. Neutral oligosaccharides are initially retained by the column and elute in order of increasing size (increasing numbers of hydroxyl groups). Oligosaccharides are detected based on their radioactivity (if previously radiolabeled) or by physical or chemical means (if unlabeled). Information about the number and size of oligosaccharide species in a mixture is thus obtained.

Materials

Oligosaccharide sample
Oligosaccharide standards (e.g., Dionex or Oxford Glycosystems)
HPLC-grade water
HPLC-grade acetonitrile

HPLC apparatus capable of two-component gradient formation
Bonded amine HPLC column (e.g., Varian Micropak AX-5 or Rainin LC-NH_2)

Additional reagents and equipment for exoglycosidase digestion (optional; UNITS 17.13 & 17.18), and reduction and desalting of neutral oligosaccharides (see Support Protocol)

1. Reduce and desalt the sample prior to analysis (see Support Protocol). If desired, prepare parallel samples treated by sequential or combined exoglycosidase digestion (UNIT 17.13 & 17.18) chosen to help elucidate the structure.

   Reduction of samples gives sharper peaks and better baseline resolution.

   The column is also a weak anion exchanger. Salts or buffers can compete with the oligosaccharide for binding sites, altering elution time and resolution. Anionic oligosaccharides will bind to the column and will not elute with water. However, the method can be adapted to study anionic oligosaccharides.

2. Equilibrate the column in 70% acetonitrile/30% water at a flow rate of 1 ml/min.

   Water and acetonitrile should be prefiltered through a 0.45-µm filter and degassed. See UNIT 10.12 support protocol for preparation and degassing of solvents. Column prefilters and/or guard columns provide additional protection and increase the productive lifetime of the column.

   All common neutral oligosaccharides should bind to the column in 70% acetonitrile. If an oligosaccharide fails to bind, the starting concentration of acetonitrile should be increased.

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3. Dissolve the sample in a small volume of water and add 3 vol acetonitrile.

   The main factor determining the appropriate sample volume is the size of the injection loop. Injection loops of ≤500 µl are preferred.

4. Load and inject the sample. Immediately start a 70% to 30% acetonitrile gradient over 80 min, maintaining the flow rate of 1 ml/min.

   Typical neutral N-linked oligosaccharides elute at water concentrations of <70%. A second gradient of 30% to 0% acetonitrile over 30 min may be included to elute unexpectedly large neutral oligosaccharides and to help keep the column clean.

5. Detect the oligosaccharides by physical, chemical, or radiometric methods. For analytical work, in-line radioactivity detectors or UV detectors may be used. If the amount of radioactive sample is low, or if the column is being used to obtain individual species for subsequent analysis, collect and analyze fractions every 0.3 to 0.5 min. For UV detection, measure absorbance at 190 to 210 nm. Determine the number of oligosaccharide species present and compare their retention times to those of known standards prepared similarly.

   Because many compounds absorb at 190 to 210 nm, UV detection demands a sample of high purity.

6. Infer features of the oligosaccharide structure from the retention time relative to standards and from changes in retention caused by sequential or combined exoglycosidase digestion.

   Because retention time is also a reflection of column performance, it is important to properly care for the column. Routine washing with water (step 4 annotation) helps remove contaminants that are hydrogen bonded to the column. Salts or other ionic contaminants can be removed by periodic washing with 0.5 M potassium phosphate, pH 1.7. Column performance will deteriorate over time, even with washing, due to gradual dissolution of the bonded phase. Standards must therefore be run at the beginning and end of each set of samples.

SUPPORT PROTOCOL

SODIUM BOROHYDRIDE REDUCTION AND DESALTING OF NEUTRAL OLIGOSACCHARIDES

Oligosaccharides with a reducing sugar at their inner terminus elute as broader peaks than those that have been reduced to alditols. Whenever possible, therefore, oligosaccharides should be reduced prior to HPLC analysis. Samples and standards should be similarly treated. Recoveries of oligosaccharides after reduction and desalting are typically ~70%. Note that a radioactive label can be introduced at this stage if ^{3}H-labeled sodium borohydride ([^{3}H]$NaBH_4$) is used (*UNIT 17.5*) and that *O*-*N*-acetyl-D-galactosamine (*O*-GalNAc)-linked oligosaccharides are reduced during their release by β-elimination (*UNIT 17.15*).

Additional Materials *(also see Basic Protocol)*

1 M sodium borohydride ($NaBH_4$) in 0.2 M sodium borate ($Na_2B_4O_7$; see recipe)
5% acetic acid/95% methanol
Mixed-bed anion and cation resin (Amberlite MB-3 or equivalent)

1. Dissolve $NaBH_4$ to 1 M final concentration in 0.2 M $Na_2B_4O_7$ (pH 9.5) immediately before use. Dissolve the dried oligosaccharides in 100 µl of this reagent and incubate 3 hr at room temperature. Because hydrogen gas may be evolved by decomposing $NaBH_4$, cautiously vent the tube occasionally or use a tall, loosely capped tube.

   Sodium borate buffer at pH 9.5 is preferred to more basic NaOH solutions, because the latter promote epimerization of the core N-glucosamine to N-mannitosamine, which can result in doublet peaks in the HPLC analysis.

2. Stop the reduction by adding dropwise a 5% acetic acid/95% methanol mixture. The sample will bubble due to evolution of hydrogen gas. Continue until no more gas is evolved.

3. Dry the sample under a stream of N_2 or using a shaker/evaporator, Speedvac evaporator, or similar device. Take to complete dryness.

4. Repeat steps 2 and 3 three times using 0.5 ml of 5% acetic acid/95% methanol each time.

   Under acidic conditions the methanol and $Na_2B_4O_7$ form volatile methyl borates. The salt left behind is sodium acetate.

5. Prewash a 2 to 5 ml column of mixed-bed resin with at least 10 column volumes of water. Dissolve the dried sample in water, load, and elute with water. Collect a single 10 to 15 ml fraction.

   Only neutral reduced oligosaccharides should pass through the column unretarded.

6. Lyophilize or dry the sample. Store the oligosaccharide at −20°C, either dry or in water.

   Presence of white powder following lyophilization indicates that not all the salt was removed by the resin. If so, repeat the desalting. A 26-G needle added to the column outlet can slow the flow and increase the time for the salts to interact with the resin. Alternatively, an anion-exchange column (e.g., Dowex 3×4a, hydroxide form, prewashed with ≥10 vol water) can be used followed by a cation-exchange column (e.g., Dowex 50, hydrogen form, prewashed with ≥10 vol water).

REAGENTS AND SOLUTIONS

Use HPLC-grade or other high-quality deionized water (e.g., 18 MΩ Milli-Q type) in all recipes and protocol steps, and filter through a 0.45-µm filter and degas before use. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

1 M sodium borohydride in 0.2 M sodium borate

Prepare 0.2 M sodium borate by dissolving 7.63 g sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) in 90 ml H_2O. Adjust pH to 9.5 with NaOH and volume to 100 ml. Store at room temperature.

Immediately before use, dissolve 37.8 mg sodium borohydride ($NaBH_4$) per ml 0.2 M sodium borate, pH 9.5. Do not mix excessively.

COMMENTARY

Background Information

The fractionation and analysis of neutral oligosaccharides on the basis of size using this HPLC method yield several important pieces of information. A minimum estimate of the number of species present is obtained from the number of peaks observed. The size of an N-linked oligosaccharide may be indicative of the extent to which it has been processed. Single or sequential exoglycosidase digestion(s) (UNIT 17.18), in conjunction with repeated HPLC analysis, yield information about the identity and linkage of the monosaccharides present. Although HPLC analysis requires more equipment than conventional chromatography on Bio-Gel P-4, the elution profiles obtained are sharper and larger oligosaccharides can be better resolved.

Bonded amine HPLC columns have free NH_2 groups that hydrogen bond to the hydroxyl groups of oligosaccharides. Elution is accomplished by increasing the water content, which progressively disrupts the hydrogen bonds. Other factors affecting elution time include the monosaccharide composition and the branching pattern of the oligosaccharide. Several methods of detecting the oligosaccharides are available. Radiolabeled molecules can be detected with an in-line radioactivity detector or by liquid scintillation counting of fractions. Oligosaccharides can be radiolabeled, either metabolically (UNIT 17.4), enzymatically (UNIT

17.6), or chemically (*UNIT 17.5*). Because some monosaccharides absorb UV light between 190 and 205 nm, they can also be detected spectrophotometrically. However, this requires quantities of oligosaccharide that are sometimes prohibitive (>5 nmol) and a sample of high purity. An alternative approach is to label the reducing terminus of the oligosaccharide with a fluorescent tag (see Tomiya et al., 1988, and references therein). However, some hydrophobic tags can produce anomalous behavior on these HPLC columns.

Interpretation of the data is dependent on the use of appropriate standards. Complex, hybrid, and high-mannose N-linked oligosaccharides and several O-linked oligosaccharide standards are commercially available. The same standards used to calibrate the HPLC column can also be used as controls for exoglycosidase digestions.

The use of bonded amine HPLC columns to separate neutral oligosaccharides was introduced in the early 1980s. Since then several articles have been published describing the behavior of model oligosaccharides on these columns and elucidating the factors that affect elution time (Mellis and Baenziger, 1981; Blanken et al., 1985). Such systems have been used to study the substrate specificity of glycosyltransferases (e.g., Schachter et al., 1989; Brockhausen et al., 1988; Koenderman et al., 1989) and to deduce the order of release of monosaccharides from oligosaccharides by exoglycosidase digestion (e.g., Tomiya et al., 1991). Sequential exoglycosidase digestion coupled with HPLC analysis has been used extensively to elucidate oligosaccharide structure (e.g., Sampath et al., 1992).

A modification of this method has been described for the fractionation of anionic oligosaccharides on the basis of size (Mellis and Baenziger, 1983). Triethylamine acetate is used to suppress the charge of sialic acid, phosphate, and sulfate, thus allowing separation of oligosaccharides on the basis of monosaccharide content alone. The reader is referred to the original description (and to *UNIT 17.17B*) for further details.

Critical Parameters

The most important parameter is column performance. There is some variability in the quality of separation by columns from different manufacturers and between columns from a given manufacturer. Decreases in column performance can be expected to occur over time due to the gradual dissolution of the bonded phase (Blanken et al., 1985). Improper care can greatly accelerate the decay of column performance. Inadequate cleaning, the use of unfiltered solvents, and the presence of contaminants in the sample can lead to higher back pressure, broader peaks, and decreased retention time.

The nature of the gradient also affects the ability of the column to resolve individual oligosaccharide species (Mellis and Baenziger, 1981). Most oligosaccharides are retained by the column if the starting acetonitrile/water ratio is 70:30. Typical gradients increase the water content by 0.5%/min. However, for separation of larger oligosaccharides the water content may need to be increased by only 0.3%/min (Blanken et al., 1985). Oligosaccharides that have not been reduced and desalted give broader peaks than reduced molecules. Reduction should be performed in sodium borate buffer and not in NaOH, as strongly alkaline conditions promote the epimerization of the GlcNAcitol to ManNAcitol, which can lead to doublet peaks (Mellis and Baenziger, 1981). Reduced oligosaccharides may have longer retention times than their unreduced counterparts (Koenderman et al., 1989). Thus, the standards used to calibrate the column should be treated the same way as the samples.

Anticipated Results

Homopolymers of increasing size of GlcNAc (chitin) and glucose (dextrans) have been separated into a series of well-spaced peaks, corresponding to oligomers that differ in size by one monosaccharide unit (Mellis and Baenziger, 1981). The elution time of oligosaccharides depends on the number of monosaccharides present and their composition. GlcNAc- and fucose-containing oligosaccharides elute sooner than oligosaccharides having an equal number of hexoses (Blanken et al., 1985), presumably because GlcNAc and fucose have one fewer hydroxyl group. The branching pattern of an oligosaccharide also affects its elution time. Oligosaccharides containing a β1-6 linkage elute later, presumably because the flexibility of this linkage allows a stronger interaction with the column (Blanken et al., 1985). Thus, it is possible to separate isomers of the same size given good column performance.

Time Considerations

A typical separation requires 1 to 2 hr, depending upon the length and steepness of the gradient and the length of time the column is

allowed to wash. At least one standard run, preferably with a mixture of standards, should be performed at the start of each day. Thus three to six samples can be analyzed in an 8-hr day.

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Key References

Blanken et al., 1985. See above.

Describes important parameters for optimal resolution of neutral oligosaccharides.

Mellis and Baenziger, 1981. See above.

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