Carbohydrates — purity assessment*

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Abstract: In this brief review a superficial introduction is given to the important area of biologically active carbohydrates. The complexity of carbohydrate chemistry is considered and the possible uses of carbohydrates as drugs and diagnostic agents are outlined. The analytical tools and methods that are especially useful for purity assessment of carbohydrates are examined with particular emphasis on liquid chromatography.

Keywords: *Carbohydrates; oligosaccharides; bioactivity; drugs; liquid chromatography; purity determination.*

Introduction

In the light of more recent research, carbohydrates have turned from uninteresting compounds used by nature for cell fuelling and structure reinforcement into highly sophisticated substances that regulate cell interaction and recognition. Generally, carbohydrates have a profound role for cell level communication in all living organisms from bacteria to humans.

These carbohydrates are usually conjugated with other molecules to form glycoproteins, glycolipids etc. The carbohydrate moiety is often a rather complicated, branched oligosaccharide but is sometimes a simple disaccharide. The protein or lipid part is anchored in the cell membrane while the carbohydrate structure stands out from the cell surface like an antenna. Different human organs have quite different sets of oligosaccharides. In addition, there are microvariations in the same organ between individuals. For example, differences in the sets of carbohydrates in the erythrocytes are responsible for different blood groups.

Carbohydrates Form Many Isomers

In complicated biological systems an enormous amount of variability is needed for communication purposes. The information density in carbohydrates is much higher than in other types of molecules. This is because oligosaccharides have three ways of generating diversity: Linkages between monosaccharides could have an α- or β-

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configuration at the anomeric carbon; linkages could be made to five different carbon atoms in a pyranose unit; and oligosaccharides could be branched.

Two monosaccharide molecules of the same type, e.g. glucose, could make as many as 11 different disaccharide isomers, but for amino acids only one peptide could be obtained. With three different monosaccharide molecules 1056 trisaccharide isomers could be formed [1] compared with six peptides for amino acids.

The oligosaccharides of a certain cell surface are specific for the tissue in question; bacteria and viruses appear to use this property for recognition of the right tissue. Bacteria can attach to the carbohydrates by means of a lectin-like substance on their fimbriae. From such a position bacteria can start to colonize and begin to produce toxin or they can penetrate the host cells.

Diagnostic and Therapeutic Possibilities

These recent discoveries open up a great number of possibilities in diagnosis and therapy. To explore these fields the first step is to get sufficient biological material of interest. Next, the right oligosaccharide has to be isolated and then its structure can be determined. Finally, by a combination of organic synthesis, biochemical methods and microbiological techniques, a sufficiently large quantity of the substance can be produced. For diagnostic purposes subkilogram quantities are usually enough. However, the development of a therapeutic drug will require quantities in the kilogram range.

For diagnostic purposes an oligosaccharide could, for example, be bonded to cover a plastic bead. Certain bacteria couple to the carbohydrate molecules with their many fimbriae to form a tight net between a number of beads. The resulting agglomerate is a visible diagnostic proof of a certain type of bacteria.

The objective of using an oligosaccharide as a drug is to supply it in sufficient quantity to block the fimbriae of the bacterium. The inactive bacteria are then simply 'washed' out of the body. The carbohydrate drug acts as an antibiotic but is non-toxic and has no pronounced side-effects.

The oligosaccharide has to be very pure for general scientific work and for use as a diagnostic agent. For use as a drug there will be additional legal requirements for purity. The general rule of thumb for drug purity to be not less that 99% should be reasonable also in this context.

Assessment of Purity

Consider the various methods of assessing the purity of a certain oligosaccharide. Generally, there is no great difference between the purity determination of carbohydrates and that of conventional types of drugs. A special difficulty, however, is the very large number of possible isomers in carbohydrate chemistry. During the synthesis of an oligosaccharide many chromatographic purification steps are carried out. Owing to the selectivity of these chromatographic processes, possible impurities in the final product are likely to be very similar to the product itself; that is, impurities will probably be isomers of the final product.

NMR spectroscopy is the main and indispensable tool for the elucidation and identification of carbohydrate structure. A proton spectrum of, for example, a tetrasaccharide is necessarily rather complicated since the molecule contains 42 hydrogen atoms. With modern high-resolution instruments the protons on the anomeric carbons

are easy to distinguish, whereas the protons on other carbons in the ring tend to merge owing to their rather similar environment. A carbon-13 spectrum is easier to interpret and signals from most carbon atoms are fairly well resolved. Impurities are usually difficult to detect below a level of 1%.

Mass spectrometry (MS) is a powerful identification tool in itself and especially to support the interpretation of a complex NMR spectrum. The limit of detection is also considerably lower than in NMR; thus MS is a better method for the detection and identification of impurities. The necessity of vaporizing the carbohydrate sample has hitherto required derivatization as in gas chromatography. Fortunately progress in the development of different direct ionization techniques has been made [2], the latest techniques being fast atom bombardment (FAB) [3, 4] and laser desorption (LD) [5].

Fourier transform infrared spectrometry (FTIR) is very handy for identification of impurities especially if a suitable reference material is available since unknown carbohydrate spectra are often difficult to interpret. Owing to the low limit of detection and computer capabilities, quantitative estimates of impurities can be made even in the nanogram range. FTIR can also be valuable to test the purity of HPLC peaks. Samples can be taken from the front and the back of a peak, and the spectra recorded; if the difference between these is zero the peak is reasonably pure. It is assumed that the retention times or peak shapes of the main and the interfering compounds are slightly different.

Figure 1
Gas chromatographic separation of trimethylsilyl derivatives of the α- and β-anomers of galactose in furanose (f) and pyranose (p) forms on a 25-m BP-10 fused silica capillary column run isothermally at 185°C.
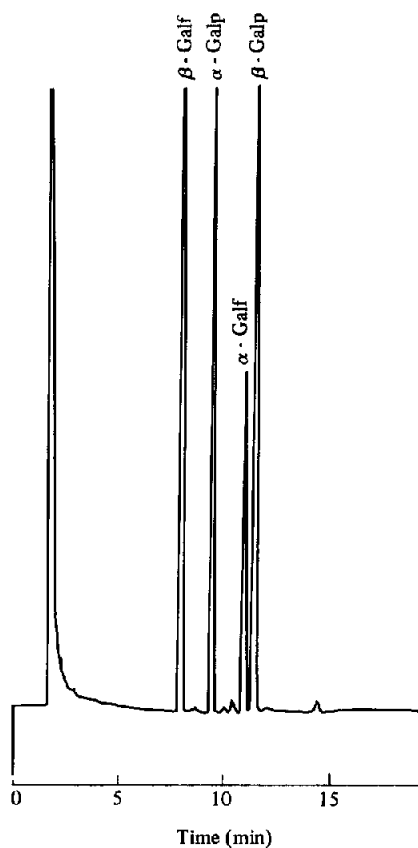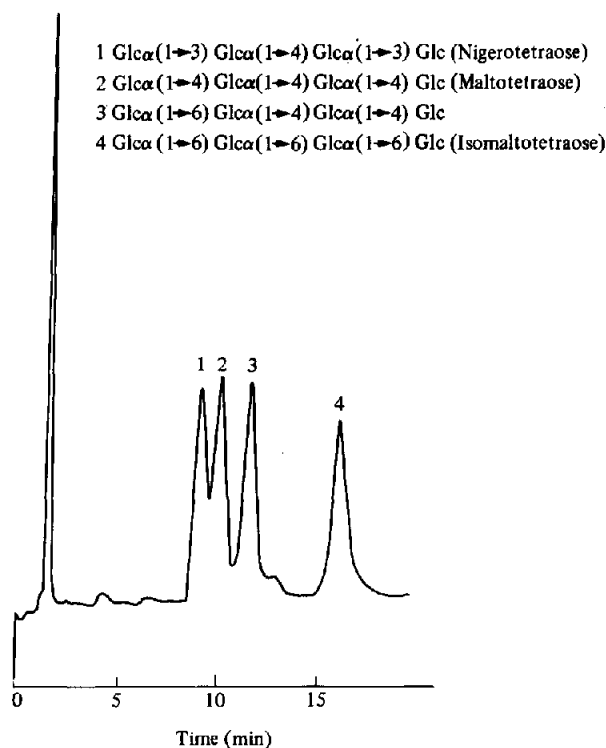
The *optical activity* of carbohydrates is used with advantage to determine overall purity. The sensitivity of modern polarimeters is high, allowing a very good measure of the purity. Very similar isomers can have very different specific rotations. For example, one glucobiose with an α 1→6 linkage compared with another one with a β 1→6 linkage have specific rotations of about +10 and +120 degrees, respectively [6, 7].

Chromatographic methods are generally very powerful means of purity assessment; 0.01–0.1% of an impurity can usually be detected. Highly specific determinations of impurities can be made with the right method. *Gas chromatography (GC)* is the traditional method in carbohydrate chemistry because it has much to offer: very low limits of detection and very good resolving power. As an example, Fig. 1 shows the separation of the α- and β-anomers of galactose in furanose and pyranose form. However, there are limitations. The low volatility of carbohydrates hampers GC in two ways. Thermal instability has to be considered and determinations are usually limited to tetrasaccharides and smaller sugars. The second consequence is the necessary derivatization process with the possibility of generating artifacts which could be misinterpreted as impurities.

Liquid Chromatography

In general, liquid chromatography (LC) is the chromatographic method of choice for carbohydrate separations. It is fast, there is no need for derivatization and detection is


1 Glcα(1→3) Glcα(1→4) Glcα(1→3) Glc (Nigerotetraose)
2 Glcα(1→4) Glcα(1→4) Glcα(1→4) Glc (Maltotetraose)
3 Glcα(1→6) Glcα(1→4) Glcα(1→4) Glc
4 Glcα(1→6) Glcα(1→6) Glcα(1→6) Glc (Isomaltotetraose)

Time (min)

Figure 2
Separation of four glucotetraose isomers on a 300 × 3.9 mm i.d. Nucleosil 5 NH$_2$ column with acetonitrile–0.01 M phosphate buffer at pH 5.9 (70:30, v/v) at a flow-rate of 2.0 ml min^{-1} and RI detection.

usually non-destructive. There are also many HPLC systems available with different selectivity allowing separation of many impurities. In addition, the option of changing eluent composition is an advantage. The most obvious disadvantage is the lower power of separation compared with that of GC. However, with the growing impact of micro-column LC, this unfavourable condition will gradually disappear.

The most common liquid chromatographic system for carbohydrate separations is aminopropyl silica with acetonitrile–water as the mobile phase. Of all available systems this usually provides the best resolution. The dominating retention mechanism seems to be liquid–liquid partition with slow mass transfer [8, 9]. Thus the plate number is usually only 10 000 or less in a 300-mm column packed with 5-μm particles. Another disadvantage of this system is that the primary amino groups react with carbonyl groups to form Schiff bases [10]. Since a small fraction of many carbohydrates is in an acyclic form, this fraction reacts with the stationary phase. The practical consequences are that these sugars show peak tailing and that the columns have a reduced life. One way of

2 - (2 - Carbomethoxyethylthio) ethyl O - α - D - glucopyranosyl -
$(1{\rightarrow}6)$ - O - α - D - glucopyranosyl - $(1{\rightarrow}4)$ - O - α - D - glucopyranosyl -
$(1{\rightarrow}4)$ - β - D - glucopyranoside
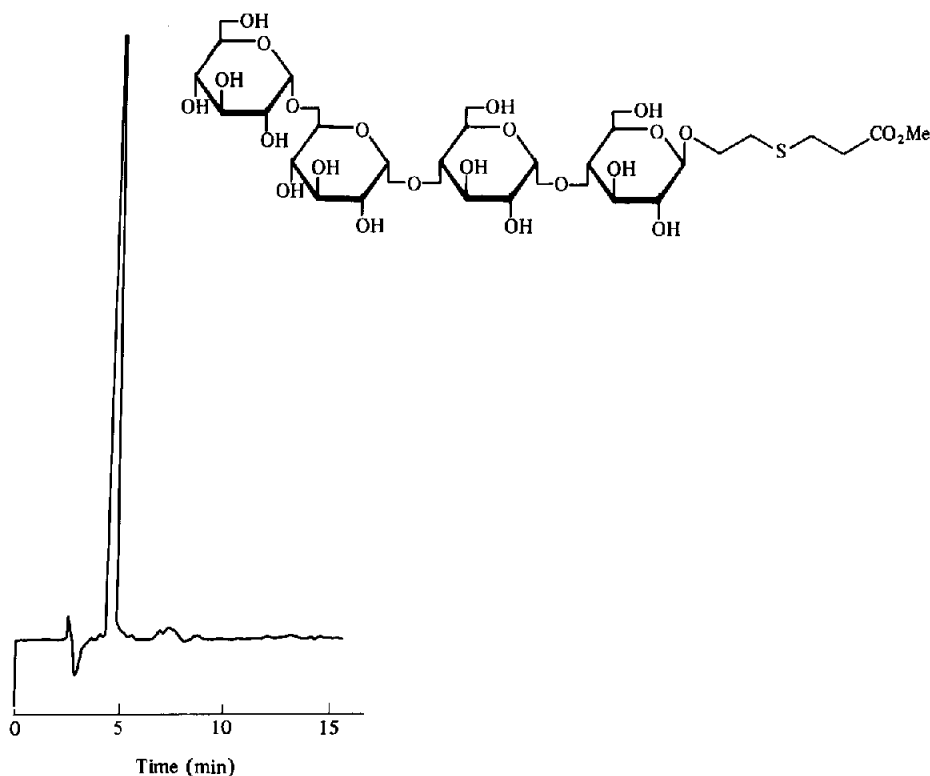
Figure 3
HPLC determination of purity of a CETE-tetrasaccharide made on a 300 × 3.9 mm i.d. Nucleosil 5 NH₂ column with acetonitrile–0.01 M phosphate buffer at pH 5.9 (60:40, v/v) at a flow-rate of 1.0 ml min⁻¹. At least nine minor impurities can be distinguished.

reducing Schiff base formation is to lower the pH of the eluent [11]. This has been done in a separation of four glucotetraose isomers as shown in Fig. 2.

The same HPLC system has been used for determination of the purity of another tetrasaccharide with a 2-(2-carbomethoxyethylthio)ethyl (CETE) spacer arm as depicted in Fig. 3.

During synthesis, saccharides are often acetylated. When in this form, sugars are easily separated on a C_{18} column with acetonitrile–water as the mobile phase [12].

Other useful phases are cation-exchange resins in, for example, calcium form [13, 14], usually run with water at an elevated temperature, 80°C being typical. One of the main retention mechanisms is size exclusion, oligosaccharides are eluted first and mono-saccharides last. Another mechanism is complex formation with the metal ion [15]. It is possible to change selectivity using other metal ions. The high eluent temperature is used to speed up mass transfer by reducing the viscosity of water and also to speed up an equilibrium displacement towards only one anomer, catalyzed by residual protons on the ion-exchange resin [14]. These two processes act to narrow the peak width.

Sometimes a C_{18} stationary phase operated with water can be useful for the separation of oligosaccharides [16]. This system has the advantage of saccharides being easily soluble in water but the disadvantage of separating carbohydrate anomers.

Refractive index (RI) detection is the most widely used detection mode for carbohydrates. Modern RI detectors are very stable and have a rather low limit of detection. UV detection from 195 to 210 nm is mainly used for gradient separations. The recently developed *mass detector* [17–19] is also especially suitable for gradient elution because of its complete insensitivity to eluent composition.

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