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Note

Separation and determination of neutral monosaccharides using methanolysis and high-peformance liquid chromatography

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The quantitative analysis of neutral monosaccharide components is of great interest when studying carbohydrates as well as glycoconjugates. This analysis is often done after acid hydrolysis followed by thin-layer chromatography[1], high-voltage electrophoresis[2] or gas chromatography[3] after necessary derivatization. Column liquid chromatography on polystyrene-based resins has also been employed[4]. The silica-based and mechanically more stable resins developed for high-performance liquid chromatography (HPLC) with polar bonded phases are valuable for the analysis of plant sugars, but some of the hexoses present in animals, such as glucose and galactose, seem difficult to separate.

Another way to degrade the polymeric carbohydrates is methanolysis, which may be preferable when studying less stable components such as the uronic acids[5], where the neutral monosaccharide components also occur in the form of methylglucosides. Since derivatives of uronic acids obtained upon methanolysis can be efficiently separated on a reversed-phase resin[6], attempts were made to separate the neutral monosaccharide components present in the same methanolysate. The aim of the present investigation was therefore to establish suitable conditions for the separation of these sugars, in particular those normally present in animals, namely the derivatives of galactose, glucose, mannose, xylose and fucose.

MATERIALS AND METHODS

A novel non-sulphated acid polysaccharide with a high content of neutral hexoses[7] was isolated from squid cranial cartilage after papain digestion followed by β-elimination, precipitation with ethanol and subsequent chromatography on DEAE-cellulose. All other chemicals used were of analytical grade.

Methanolysis was performed in sealed Pyrex glass tubes, using 1 *M* HCl in dry methanol at 100°C for 30 h[6]. In order to remove irrelevant products, such as hexosamines, the dried methanolysates were dissolved in a small volume of methanol and passed through an ion-exchange column (15 × 3 mm I.D.) of Dowex 50-X8. The

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column was then washed with 1 ml methanol, the pooled eluate containing virtually all the neutral sugar derivatives. When the preparation was expected to contain acidic components that would be esterified during methanolysis and which therefore might disturb the subsequent HPLC, a slightly modified procedure may be advisable[6]. The dried methanolysates were hydrolyzed in a 1% ammonia solution at 100°C for 3 h. After subsequent desiccation the material was dissolved in methanol and added to the 3 mm I.D. ion-exchange column, which contained 7 mm AG 1-X8 layered over 7 mm of Dowex 50-X8. The neutral sugar derivatives were eluted from this column with 1 ml of methanol.

After desiccation the material was dissolved in a small volume of eluent and centrifuged at 10,000 *g* for 5 min. Aliquots of the supernatant were added to the column via a loop injector.

The monosaccharide preparations were chromatographed on a 100 × 4.6 mm I.D. CP™-MicroSpher C_{18} column (Chrompack, Middelburg, The Netherlands) eluted with water at 0.7 ml/min. The eluted material was monitored using an Optilab 931 chromatograph with a high-sensitivity refractive index (RI) detector (15-mm cell) at just above room temperature, or by connecting the column to a Technicon AutoAnalyzer II system designed for the anthrone reaction[8].

RESULTS AND DISCUSSION

The 1-methylglycosides of most neutral sugars present in animals were eluted by water from the reversed-phase column within 3–7 min, while the less polar fucose needs 14 min to be eluted (Fig. 1). The first of the derivatives to be eluted is the main galactose glycoside, which appears very close to the solvent front together with traces

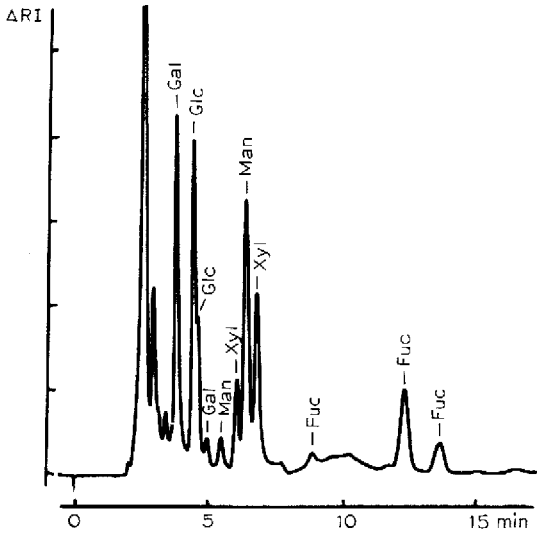

Fig. 1. Separation of a mixture containing 1-methylglycosides of galactose (Gal), glucose (Glc), mannose (Man), xylose (Xyl) and fucose (Fuc) on a 100 × 4.6 mm I.D. CP-MicroSpher C_{18} column. The peaks correspond to 1 μg of each monosaccharide added to the column. The column was eluted with water at 0.7 ml/min. The duplicate peaks from each monosaccharide may represent anomeric forms.

of methanol. In order to obtain adequate conditions for determination of galactose it is therefore necessary to evaporate all methanol from the sample before dissolving it in the eluent for the subsequent chromatography. When attempts were made to study a methanolysate of sucrose, only the glucose glycoside peak was obtained, indicating that the fructose glycoside had been hidden by a strongly negative peak due to traces of methanol. Two peaks were obtained for most of the neutral sugars studied, possibly due to the existence of anomeric forms. This was, however, not further investigated.

When radioactively labelled glucose was employed, 93% of the methanolyzed material was recovered as the 1-methylglycoside, while the remaining 7% appeared closer to the front together with unreacted hexoses. This is in contrast to the situation with uronic acids, where the recovery upon methanolysis is much lower. It is therefore necessary to use external standards when the amounts of neutral sugars and uronic acids in the same methanolysate are to be compared, thereby compensating for specific losses of uronates.

With the high recovery of the neutral sugar derivatives, it is not surprising that the peak heights obtained were linearly related to the amounts methanolyzed. The calibration curve was linear over a very wide concentration range, with no signs of column overloading even at full scale deflection with the highest detector attenuation ($\times$ 100). With the RI detector, the detection limit, expressed as twice the baseline noise, corresponded to less than 25 ng of glucose, thus allowing the determination of 500 ng within a 5% confidence interval. The use of UV detection at low wavelengths was also tested, but the sensitivity was too low. The AutoAnalyzer II system used exhibited a sensitivity similar to that of the RI measurements, but the resolution between closely eluting peaks such as glucose and galactose was poor. These sensitivities were obtained with a 3-μm-particle resin. Similar results could be obtained with other kinds of octadecylsilane (ODS) columns. A theoretical plate count exceeding 7000 was, however, necessary, and to obtain this with a coarser resin a longer column had to be used. With some different 5-μm ODS resins it was necessary to use 250–400 mm long columns to obtain the same separation. However, due to peak dilution with the larger column volumes the sensitivity was correspondingly decreased. The choice of ODS resin thus does not seem to be of major importance for the separation, as long as the theoretical plate count is sufficient.

When the procedure was employed to analyze lactose and the novel polysaccharide isolated from squid cranial cartilage the chromatograms given in Fig. 2 were obtained. No attempts were made to integrate peak areas, but when the chromatograms were compared to external standards the lactose preparation was shown to contain a 1:1 ratio of galactose to glucose, while the squid polysaccharide was shown to contain galactose and mannose in a 2:1 ratio and smaller amounts of glucose.

The presented HPLC procedure is thus a very simple technique for the separation and determination of neutral monosaccharides. Since the same methanolyzates can be used for the determination of the uronic acid composition[6], both these classes of substances can be analyzed with only a small sample consumption. With the high sensitivity for neutral sugar components, one possible application could be to the determination of average molecular weights for glycosaminoglycans such as chondroitin sulphates, where every chain has one protein linkage region consisting of one xylose and two galactose units. From the galactose (or xylose) to uronic acid ratios the average molecular size on a number basis ($\bar{M}_n$) is therefore easily calculated.

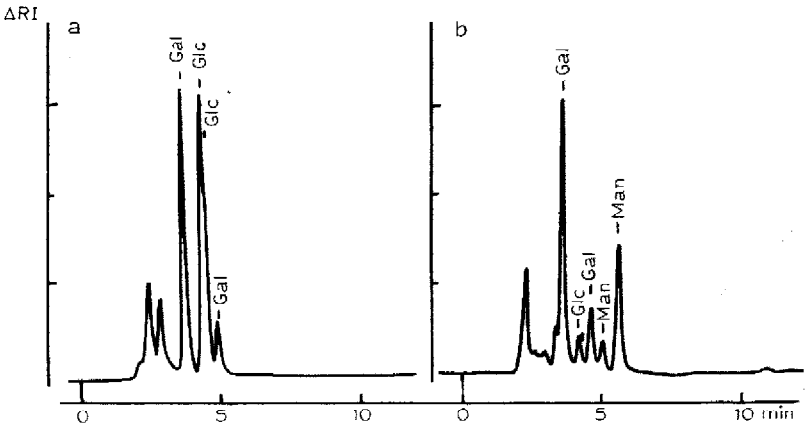

Fig. 2. Analyses of lactose (a) and the non-sulphated polysaccharide isolated from squid cranial cartilage (b). Abbreviations and conditions as in Fig. 1.

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