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A METHOD FOR THE MICROANALYSIS OF HEXOSES IN GLYCOPROTEINS

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ABSTRACT

A procedure has been developed by which $<\mu$g quantities of hexoses in glycoproteins can be determined by g.l.c.–m.s. with selected-ion monitoring of the alditol acetates derived from the sugars released by acid hydrolysis. The effectiveness of the method was demonstrated by determination of the hexose and hexosamine composition of 5-μg samples of antithrombin III and von Willebrand factor, respectively.

INTRODUCTION

The oligosaccharide moieties play an important role in many of the biological functions of the glycoproteins[1]. Glycoproteins from humans contain *O*- and *N*-linked oligosaccharides which generally show a high degree of structural heterogeneity. In the *N*-linked oligosaccharides, L-fucose, D-mannose, D-galactose, and 2-acetamido-2-deoxy-D-glucose are, and *N*-acetylneuraminic acid may be, present[1], whereas 2-acetamido-2-deoxy-D-galactose is found only in the *O*-linked oligosaccharides and, thus, is of importance for their detection and quantification.

The release of monosaccharides by acid hydrolysis, followed by identification and quantification of the derived alditol acetates[2] by g.l.c., has been used extensively in studies of the structures of glycoconjugates. When only small quantities of glycoproteins are available, methods are required for the analysis of the component sugars in μg quantities. G.l.c. analysis of the products derived by methanolysis followed by trifluoroacetylation[3] or by hydrolysis with 4M trifluoroacetic acid followed by conversion of the free sugars into *O*-methyloxime acetates[4] has been described. However, in these procedures, more than one derivative is formed from each sugar, thereby making the chromatograms complicated and quantification

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difficult. H.p.l.c. with u.v. detection is a more sensitive method for the analysis of neutral sugars as their phenyl isocyanate[5] or dansylhydrazine[6] derivatives; with the latter method, it was possible to analyse 125 μg of glycoprotein that contained ~5% of carbohydrate. Pulsed amperometric detection, in combination with anion-exchange chromatography[7,8] at high pH, gives good selectivity for carbohydrates, as reducing sugars, oligosaccharides, and alditols. Good sensitivity and selectivity of alditol acetates[9] and partially methylated alditol acetates[10,11] have been obtained by g.l.c.–m.s. with selected-ion monitoring. We now report a procedure by which hexoses and hexosamines, in quantities down to 50 ng in glycoproteins, can be determined by conversion of the released hexoses into alditol acetates followed by g.l.c.–m.s. with selected-ion monitoring.

EXPERIMENTAL

General methods. — All solvents used were of analytical grade. Teflon tubes (10 mL; Nalgene), which could be sealed with screw-caps of teflon, were used for the acid hydrolyses. The glass line of the g.l.c. injector was treated with 5% chlorodimethylsilane in heptane, then kept overnight at 100° before use.

Human antithrombin III[12] and von Willebrand factor[13] were obtained as described.

Preparation of standards. — L-Fucose, D-mannose, D-galactose, and 2-amino-2-deoxy-D-galactose·HCl (~20 mg each) and 2-amino-2-deoxy-D-glucose·HCl, 2-amino-2-deoxy-D-mannose·HCl, and *myo*-inositol (~35 mg each) were each dissolved in water (100 mL); the last two compounds were used as internal standards.

Standard solutions were prepared using 0 (S0), 0.5 (S1), 1.0 (S2), 2.0 (S3), and 4.0 mL (S4) of the respective sugar solution. Solutions of *myo*-inositol (6.0 mL) and 2-amino-2-deoxy-D-mannose (4.0 mL) were added to each, and then water to make the volume up to 100 mL. Portions (2 mL) of these solutions were stored in sealed tubes at −20°. The solutions were stable for 1 week at 4°.

Sugar analysis. — Standard S0 (50 μL) was added to an aqueous solution of antithrombin III or von Willebrand factor (5–10 μg in 0.45 mL) in a 10-mL teflon tube, and trifluoroacetic acid (0.11 mL) was added to give 2.3M acid. Tubes were also prepared containing standard (S0–S4; 50 μL), trifluoroacetic acid (0.11 mL), and water to a final volume of 0.61 mL. The sealed tubes were heated for 2 h at 120°, then cooled to room temperature, and the contents of each tube were transferred to a glass tube (12 mL) and kept at 40° (bath) whilst the solvents were removed by a stream of air. A solution of sodium borohydride (10 mg.mL^{-1}) in M ammonia (0.2 mL) was added to each tube, which was then kept for 30 min at 60°, more (0.2 mL) of the solution of sodium borohydride was added, and the solution was left for 15 min at 60°. The temperature was raised to 100°, the solvents were removed by a stream of air, 0.7M hydrochloric acid in methanol (2 mL) was added, and the solvents were removed as described above. Another portion (2 mL) of 0.7M hydrochloric acid in methanol was added and the procedure was repeated.

Water (50 μL) was added to each tube which was heated to 100° and shaken or sonicated to give a clear solution. Acetic anhydride (0.5 mL) followed by pyridine (0.5 mL) was added to each tube, and the sealed tubes were heated at 125° for 2 h, then cooled to room temperature. Water (6 mL) was added to each tube followed by dichloromethane (3 mL), the mixture was shaken for 15 min, and the organic phase was washed with 0.2M sodium hydroxide (5 mL) then with water (5 mL), filtered immediately through silanized glass-wool in a Pasteur pipet into a centrifuge tube (NS 14) in order to remove the last traces of water, and then concentrated at room temperature. Using an automatic sampler, portions (1 μL) of a solution of each residue in toluene (100 μL) were subjected to g.l.c.–m.s.

In a separate experiment, von Willebrand factor (6.1 μg) was added to each sugar standard, and the samples were hydrolysed, worked-up, and analysed as described above.

G.l.c.–m.s. with selected-ion monitoring. — An HP 5970B mass selective detector (Hewlett–Packard) was used. Alditol acetates were separated on a fused-silica capillary column (25 m × 0.2 mm, cross-linked methylsilicon, film-thickness 0.5 μm, Hewlett–Packard). Samples (1 μL) were injected in the split-less mode (2 min), using an automatic sampler (Hewlett–Packard 7673A). An initial oven temperature of 120° was used, which was increased immediately at 25°.min^{-1} to 250° and then kept thereat.

The ions used for selected monitoring were *m/z* 187 (6–8 min) for 6 deoxyhexoses, 187 and 199 (8–10 min) for hexoses and inositol, 139 and 144 (10–12 min) for hexosamines. A selected-ion mass chromatogram was constructed by addition of the responses for the ions monitored. The ions with *m/z* 187 and 199 were sampled twice in order to obtain similar peak heights for the neutral sugars and the amino sugars, as two ions were sampled for the latter. A dwell time of 15 μs was used for the selected-ion monitoring, which gave a frequency of data points sufficient to enable quantification by comparison of peak-heights.

Calibration curves were constructed from the peak-height ratios (H) versus weight ratios (W) for the sugar standards, S0–S4, (hexose/inositol and amino sugar/2-amino-2-deoxymannose). Quantifications were made by Excel (Microsoft) and the calibration curves were drawn by Cricketgraph (Cricket Software) using a Macintosh SE/30.

RESULTS AND DISCUSSION

In the procedure described above for the analysis of sugars, some of the steps are modified, compared to the procedures used generally, in order to obtain high yields of products when <μg amounts of sugars in glycoproteins were used for analysis. Optimal yields of monosaccharides were obtained by hydrolysis with 2.3M trifluoroacetic acid for 2 h at 120°. Sealed teflon tubes gave better reproducibility of the yields of the different sugars than when the hydrolyses were performed in glass vials.

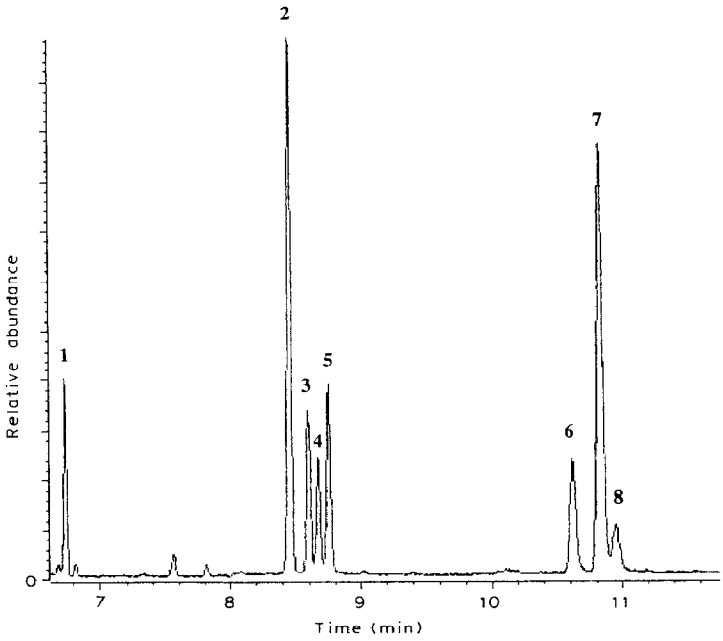

Fig. 1. The reconstructed selected-ion mass chromatogram obtained from sugar analysis of standard S2. The peaks correspond to derivatives of L-fucose (1), *myo*-inositol (2), D-mannose (3), D-glucose (4), D-galactose (5), 2-amino-2-deoxy-D-glucose (6), 2-amino-2-deoxy-D-mannose (7), and 2-amino-2-deoxy-D-galactose (8).

Hydrochloric acid in methanol was used instead of acetic acid in order to decompose excess of borohydride and to remove the boric acid, since this caused less problems in the subsequent acetylation step. The alditols and the salt were dissolved in a small amount of water before acetylation to obtain reproducible and quantitative acetylation of the alditols. Partitioning of the acetylated products between dichloromethane and water and 0.2M sodium hydroxide removed most of the acetylation reagents and products from the degraded protein. This procedure saved syringes and the injector glass line from contaminations, gave reproducible results, and prolonged the lifetime of the capillary column.

The hexose and hexosamine compositions could be determined accurately in quantities at least down to 50 ng of each sugar. The standard deviation for the quantification of the hexoses was 10%, using 10 μg of glycoprotein for the analysis.

A reconstructed selected-ion mass chromatogram obtained during analysis of the standard S2 is shown in Fig. 1. Good resolution of all the sugar derivatives was obtained within 12 min. Various amounts of glucose were detected. Glycoproteins, isolated from columns of Sephadex, had increased contents of glucose.

A plot (Fig. 2) of peak-height ratios (H) obtained by selected-ion monitoring versus weight ratios (W) for the different sugars was linear in the range 50–400 ng. A linear response was obtained also when von Willebrand factor (6.1 μg) was added to each sugar standard before hydrolysis.

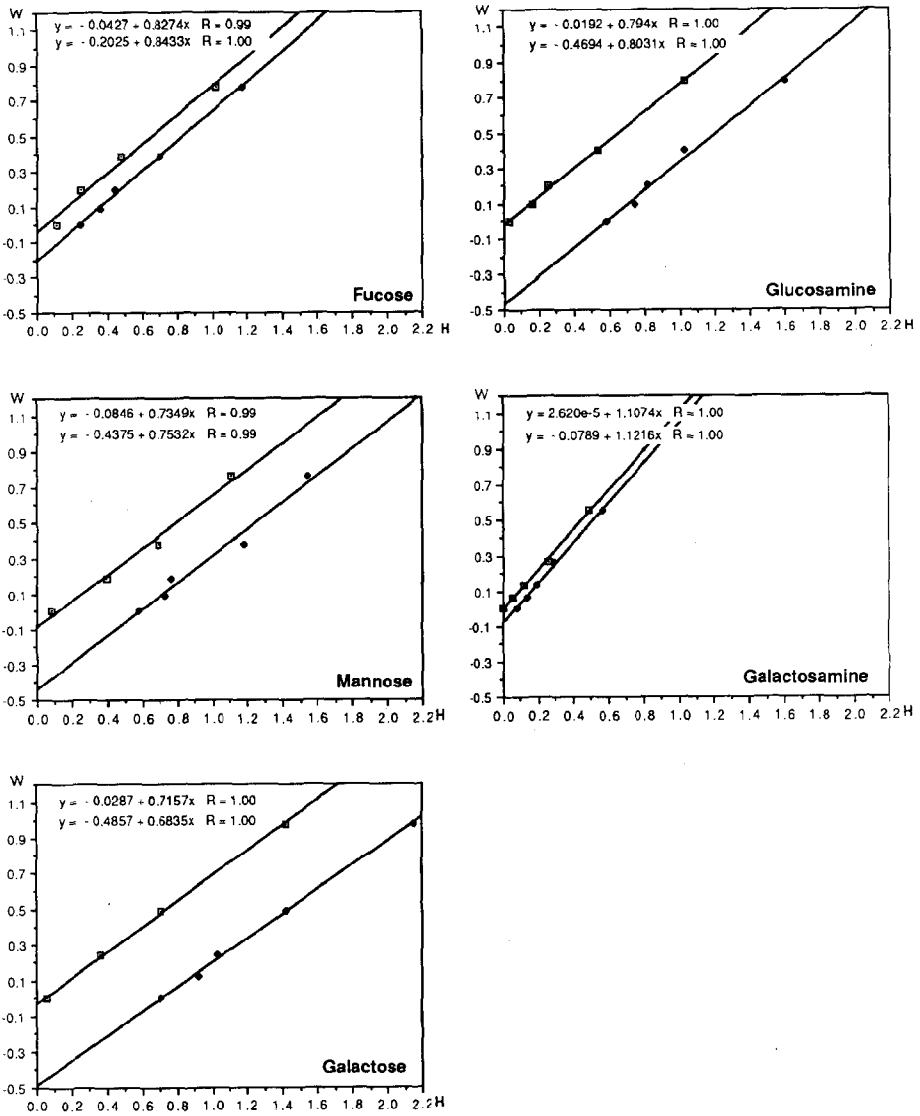

Fig. 2. Peak-height ratios (H) obtained by selective ion monitoring versus weight ratios (W) after hydrolysis of the standards with (◆) and without (□) von Willebrand factor (6.1 μg). (Negative values for W are obtained when background peaks occur).

Good selectivity and sensitivity were obtained by g.l.c.–m.s. with selected-ion monitoring. Abundant fragments in the higher mass range were selected in order to minimize interference. The peak at *m/z* 144 for the alditol acetate from 2-amino-2-deoxygalactose had an intensity lower than that from the corresponding derivative of 2-amino-2-deoxyglucose, but the intensities of the peaks at *m/z* 139 were

reversed so that both these ions were monitored. Using the standard curves for S0–S4, all the sugars could be quantified.

α-NeuNAc-(2→6)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→2)-α-D-Man*p*
1
↓
6
β-D-Man*p*-(1→4)-β-D-GlcNAc*p*-(1→4)-β-D-GlcNAc*p*-(1-N)-Asn
3
↑
1
α-NeuNAc-(2→6)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→2)-α-D-Man*p*
1

The method was applied to antithrombin III, which contains four identical *N*-linked oligosaccharides[14] (**1**), corresponding to 9.4% of hexoses. Sugar analysis (Fig. 3) showed that the 8.5% comprised Man, Gal, and GlcN in the molar ratios 3.0:2.2:5.0. These data accord with previous results[14].

The method was also applied to von Willebrand factor, which contains[15] 6.5% of hexose and 8.7% of 2-acetamido-2-deoxyhexose. The results obtained (Fig. 4) with 6.3 μg of the glycoprotein indicated Fuc 1.8, Man 4.0, Gal 4.0, GlcNAc 5.2, and GalNAc 0.9%, which corresponded to 9.8% of Hex and 6.1% of HexNAc. The earlier results were based on the phenol–sulfuric acid method for total neutral sugars and a modified Elson–Morgan method for total amino sugars. Different sugars have different responses in these methods.

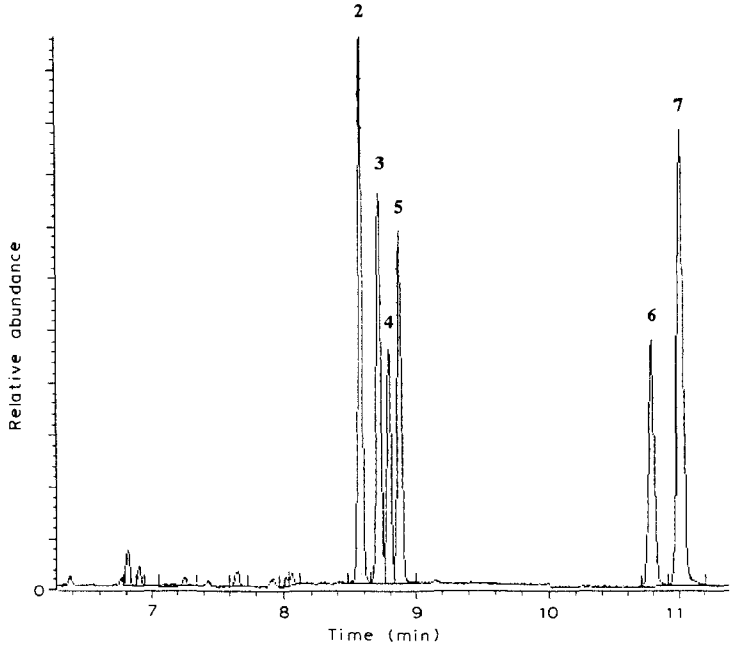

Fig. 3. The reconstructed selected-ion mass chromatogram obtained from sugar analysis of human antithrombin III (5 μg): 2–7 as in Fig. 1.

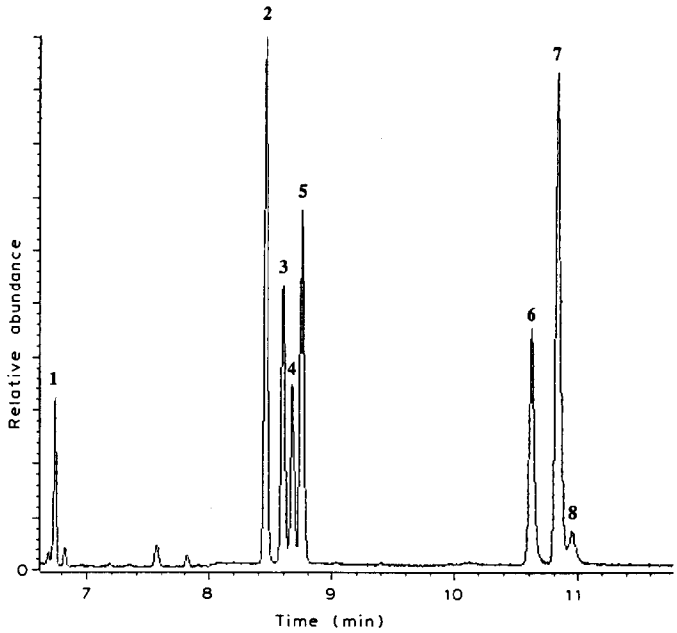

Fig. 4. The reconstructed selected-ion mass chromatogram obtained from sugar analysis of human von Willebrand factor (5 μg): 1–8 as in Fig. 1.

The method has now been applied to a glycosylated form of insulin-like growth factor (IGF-I), produced by yeast, and the tissue plasminogen activator (t-PA) obtained from human melanoma cells. The results accorded with those obtained using ^{1}H-n.m.r. spectroscopy.

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