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SEPARATION OF SIALYL-OLIGOSACCHARIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO THE ANALYSIS OF MONO-, DI-, TRI- AND TETRASIALYL-OLIGOSACCHARIDES OBTAINED BY HYDRAZINOLYSIS OF α_1-ACID GLYCOPROTEIN

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SUMMARY

High-performance liquid chromatography has been applied to the separation of isomers of mono-, di-, tri- and tetrasialylated oligosaccharides derived from α_1-acid glycoprotein by hydrazinolysis. The separation of the sialyl-oligosaccharides on the basis of their negative charges was carried out with quaternary amine-bonded silica. Within each class, the anionic oligosaccharides were fractionated on the basis of their net carbohydrate content on alkylamine-modified silica using a mobile phase consisting of a mixture of acetonitrile and potassium dihydrogen phosphate with 0.01% of 1,4-diaminobutane or 0.01% of tetraethylpentamine.

INTRODUCTION

Specific cleavage of carbohydrate chains from glycoproteins and glycopeptides provides a useful means for structural studies of glycans. The resulting oligosaccharides may be separated and purified by high-performance liquid chromatography (HPLC). Treatment with sodium borohydride in the presence of sodium hydroxide[1] allows oligosaccharide alditols to be obtained from glycan chains O-glycosidically linked to serine and threonine. Hydrazinolysis[2] results in the liberation of glycans N-glycosidically bound to an asparagine residue and in N-deacetylation of N-acetylhexosamine and N-acetylneuraminic acid. Many studies of this procedure have shown that the N-acetylglucosamine residue linked to asparagine gives several compounds, increasing the number of oligosaccharide isomers[3–5]. More recently, Nilsson and Svensson[6,7] have developed a procedure to cleave both N- and O-glycosidic linkages in which the glycoprotein is treated with a mixture of trifluoroacetic acid and trifluoroacetic anhydride.

More specific cleavages are achieved by enzymatic hydrolysis. Two different

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classes of enzymes are used: endo-N-acetylglucosaminidase, which cleaves in the pentasaccharidic core of glycoprotein between the two N-acetylglucosamines[8,9], and a glycopeptidase which splits the linkage between the first N-acetylglucosamine and the asparagine residue[10].

Many oligosaccharides liberated by cleavage techniques such as those described here possess sialic acid residues in non-reducing terminal positions. The liquid chromatographic separation of acidic oligosaccharides has been described: mucine-derived saccharides by Bergh *et al.*[11,12]; alkali-labile sialylated oligosaccharides from Cad glycophorin A by Herkt *et al.*[13] and cervical mucus oligosaccharides by Lamblin *et al.*[14]. Recently, Paz Parente *et al.*[15] described the fractionation of high-molecular-weight monosialyl-oligosaccharides isolated by hydrazinolysis from hen ovomucoid and demonstrated that the addition of 0.01% of 1,4-diaminobutane to the potassium dihydrogen phosphate solution increases the separation on primary amine-bonded silica.

In this paper we describe methods for the HPLC of high-molecular-weight acidic oligosaccharides obtained by hydrazinolysis of α_1-acid glycoprotein on a bonded primary amine packing with solvents containing 1,4-diaminobutane (DAB) or tetraethylenepentamine.

EXPERIMENTAL

Glycoprotein and oligosaccharides

α_1-Acid glycoprotein from human plasma was prepared by ammonium sulphate precipitation according to Weimer *et al.*[16], followed by cation-exchange chromatography on XE-64 as described by Schmid *et al.*[17]. Oligosaccharides were released from 500 mg of α_1-acid glycoprotein by hydrazinolysis as described previously[18]. They were N-reacetylated according to Reading *et al.*[19] and reduced with potassium borohydride.

Purification of oligosaccharides by gel filtration

Oligosaccharides were purified by gel filtration on a column (80 × 1.9 cm I.D.) of Bio-Gel P-2 (200–400 mesh, Bio-Rad Labs.) eluted with water at a flow-rate of 12 ml/h. The volume of each fraction was 4 ml.

HPLC separation of oligosaccharides from Bio-Gel P-2 fraction[20]

HPLC was performed on a 10-μm MicroPak AX-10 column (50 × 0.8 cm I.D., Varian) with a Spectra-Physics Model 700 liquid chromatograph equipped with a Model 8400 variable-wavelength detector connected to a Model 4100 computing integrator.

For preparative chromatography, 20 mg of the Bio-Gel P-2 fraction dissolved in 60 μl of distilled water were subjected to HPLC using a gradient of 500 m*M* potassium dihydrogen phosphate (adjusted to pH 4.0 with phosphoric acid) as follows: elution with distilled water for 5 min; linear gradient to 2.5% potassium dihydrogen phosphate (500 m*M*) for 15 min; isocratic elution for 25 min; linear gradient to 5% potassium dihydrogen phosphate (500 m*M*) for 35 min; isocratic elution for 45 min and linear gradient to 40% potassium dihydrogen phosphate (500 m*M*) for 40 min. The flow-rate was 2 ml/min. The oligosaccharides were detected at 200 nm

with a detector sensitivity of 0.32 and an integrator attenuation of 16. The chart speed of the integrator was 0.5 cm/min.

Each fraction was purified by gel filtration on Bio-Gel P-2 as described previously. The fractions were revealed with orcinol–sulphuric acid reagent[21] on thin-layer plates of pre-coated silica gel 60 (Merck). The phosphate salts eluted from the Bio-Gel column were identified by precipitation with silver nitrate.

HPLC separation of monosialyl-oligosaccharides (F-1) and disialyl-oligosaccharides (F-2) on primary amine-bonded silica

HPLC was performed on three columns in series: two columns of 5-μm amino AS 5A (25 × 0.4 and 15 × 0.4 cm I.D., Chromatem 33; Touzard et Matignon) and one 3 μm amino column (7.5 × 0.4 cm I.D., Chromatem 33). The columns were equilibrated with the initial solvent (solvent A) consisting of a mixture of acetonitrile (70%) and 50 m*M* potassium dihydrogen phosphate (30%). 100 μl of DAB were added per litre of solvent A. Solvent B was water. The solvents and columns were heated at 40°C. A 1-mg amount of oligosaccharides dissolved in 10 μl of solvent A was injected into the columns. After the injection, isocratic conditions with 100% of solvent A were applied for 30 min, followed by a linear gradient to solvent B–water (85:15) for 60 min and then isocratic conditions for 60 min. The flow-rate was 1 ml/min. The oligosaccharides were detected at 200 nm with a detector sensitivity of 0.16 and an integrator attenuation of 16. The chart speed of the integrator was 0.5 cm/min.

Each collected fraction containing oligosaccharides and salts was purified by rapid gel filtration on a column (25 × 1.6 cm I.D.) of Bio-Gel P-6 DG (90–180 μm, Bio-Rad Labs.) using a Spectra-Physics Model 8770 liquid chromatograph with a 500-μl sample loop. The oligosaccharides were detected with an UV detector (LKB 2138 Uvicord S) at 206 nm. The flow-rate of distilled water was 0.4 ml/min.

HPLC separation of trisialyl-oligosaccharides (F-3) and tetrasialyl-oligosaccharides (F-4) on primary amine-bonded silica

HPLC was performed on three alkylamine columns in series (see above) at 50°C to avoid precipitation of phosphate salts in the column. A 1-mg amount of oligosaccharides F-4 dissolved in 10 μl of the eluent was injected into the columns. The columns were equilibrated with a mixture of acetonitrile (60%) and 200 m*M* potassium dihydrogen phosphate (40%); 100 μl of tetraethylpentamine were added per litre of solvent. The solvents and columns were heated at 50°C and the chromatography was carried out under isocratic conditions for 160 min. The flow-rate was 1 ml/min. The oligosaccharides were detected at 200 nm with a detector sensitivity of 0.08 and an integrator attenuation of 8. The chart speed of the integrator was 0.5 cm/min. Each collected fraction containing oligosaccharides, potassium dihydrogen phosphate and tetraethylpentamine was purified by rapid gel filtration on Bio-Gel P-6 DG (see above).

Molar composition of oligosaccharides

The molar composition of oligosaccharides was determined by gas–liquid chromatography (GLC) of trifluoroacetylated methyl glycosides according to Zanetta *et al.*[22].

Permethylation

Permethylation of the oligosaccharide F-I-4 was performed according to Paz Parente *et al.*[23] with lithium methyl sulphinyl carbanion and methyl iodide, and the partially methylated monosaccharides were identified by GLC–mass spectrometry (MS) as described by Fournet *et al.*[24].

RESULTS AND DISCUSSION*

The purification of the hydrazinolysate of 500 mg of α_1-acid glycoprotein gives 190 mg of oligosaccharide alditols with a yield of 93%. The carbohydrate composition was determined by GLC (Table I). No difference was observed in the molar ratio of the total carbohydrate fraction from α_1-acid glycoprotein and that of the glycans released by hydrazinolysis; in particular, the molar ratio of sialic acid is the same, indicating that the hydrazinolysis procedure does not lead to desialylation.

TABLE I

CARBOHYDRATE COMPOSITION AND WEIGHT OF THE FRACTION OBTAINED BY BIO-GEL P-2 CHROMATOGRAPHY OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS OF α_1-ACID GLYCOPROTEIN

Fraction	*Molar ratio**					*GlcNAc/ Man*	*Weight (mg)*
	Fuc	*Gal*	*Man*	*GlcNAc*	*NeuAc*		
α_1-Acid glycoprotein	0.47	3.6	3	4.4	3.4	1.46	500
Hydrazinolysate after Bio-Gel	0.57	3.65	3	4.5	3.5	1.5	190

* The molar ratio of mannose (Man) was taken as 3.

HPLC on a quaternary amine packing of the carbohydrate fraction obtained by Bio-Gel P-2 chromatography of the hydrazinolysate of α_1-acid glycoprotein gives four fractions corresponding to the mono-, di-, tri- and tetrasialylated oligosaccharides. These fractions were obtained in 60 min. The results of the preparative chromatography of 190 mg of oligosaccharides and the carbohydrate compositions of the fractions are given in Table II. The use of the semi-preparative AX-10 column provides a quantitative recovery (91%) of the oligosaccharides. No trace of neutral oligosaccharides was obtained, demonstrating that in native α_1-acid glycoprotein all the glycan chains are sialylated. This glycoprotein comprises 5.8% of monosialylated glycans, 34.6% of disialylated glycans, 43.3% of trisialylated glycans and 16.2% of tetrasialylated glycans. For each fraction, the ratio GlcNAc/Man indicates that the number of branches linked to the trimannosido core is less or equal to four, as demonstrated previously[25–28].

* Abbreviations: Fuc = fucose; Gal = galactose; Man = mannose; GlcNAc = N-acetylglucosamine; GlcNAc-ol = N-acetylglucosaminitol; NeuAc = N-acetylneuraminic acid.

TABLE II

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY PREPARATIVE CHROMATOGRAPHY OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS OF α_1-ACID GLYCOPROTEIN

Fraction	*Molar ratio**					*GlcNAc/ Man*	*Weight (mg)*
	Fuc	*Gal*	*Man*	*GlcNAc*	*NeuAc*		
Total oligosaccharides	0.57	3.65	3	4.5	3.5	1.5	190
F-I Monosialyl-oligosaccharides	0.25	2	3	3.2	1.1	1.06	10
F-II Disialyl-oligosaccharides	0.25	2.5	3	3.5	2.2	1.16	60
F-III Trisialyl-oligosaccharides	0.6	3.3	3	4.4	3.2	1.46	75
F-IV Tetrasialyl-oligosaccharides	0.7	4.3	3	5.2	3.9	1.73	28
Recovery							173

* The molar ratio of mannose (Man) was taken as 3.

The monosialylated fraction (F-I) and the disialylated fraction (F-II) from the AX-10 chromatography were subjected to HPLC using acetonitrile–potassium dihydrogen phosphate–DAB on alkylamine-modified silica. Fourteen fractions from the monosialylated fraction (Fig. 1) and also from the disialylated fraction (Fig. 2) were obtained. The carbohydrate compositions and weights of each fraction are given in Tables III and IV. The carbohydrate composition of the monosialylated oligosaccharides indicates four classes of oligosaccharides: biantennary (class A), triantennary (class B), monofucosylated triantennary (class BF) and a new class not pre-

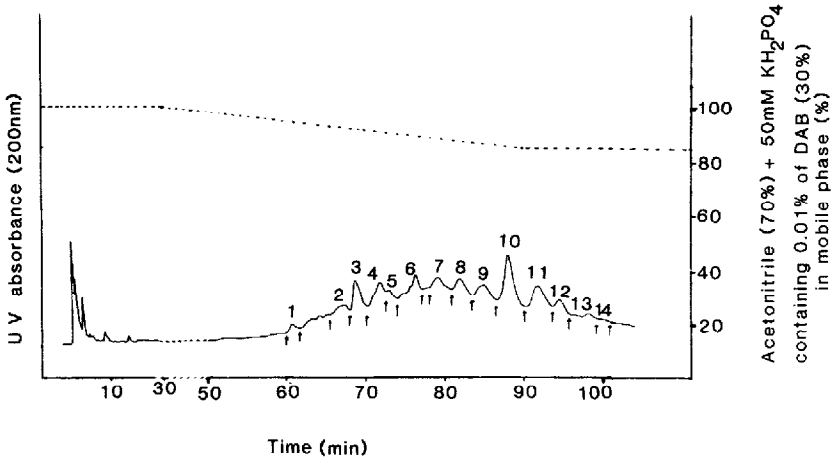

Fig. 1. Analysis of monosialyl-oligosaccharides from the hydrazinolysis of α_1-acid glycoprotein F-I by liquid chromatography on primary amine-bonded silica with acetonitrile–potassium dihydrogen phosphate–DAB as eluent.

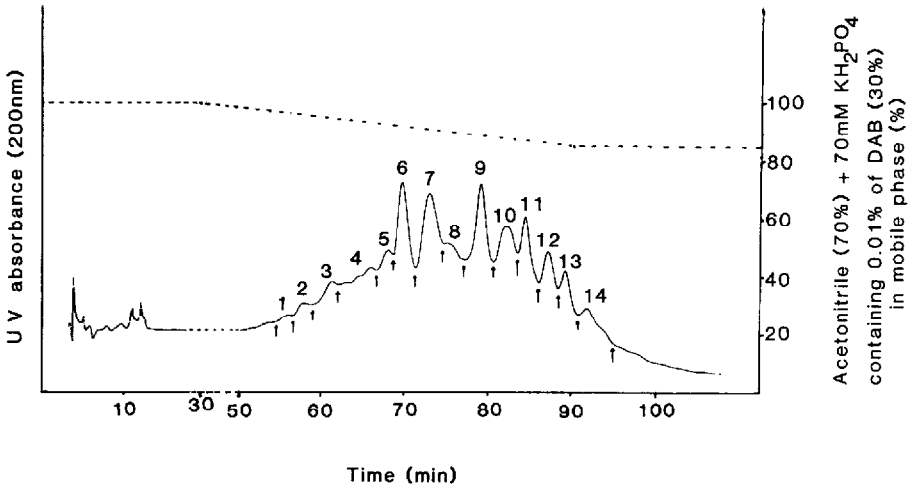

Fig. 2. Analysis of disialyl-oligosaccharides from the hydrazinolysis of α_1-acid glycoprotein F-II by liquid chromatography on primary amine-bonded silica with acetonitrile–potassium dihydrogen phosphate–DAB as eluent.

TABLE III

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF MONOSIALYLATED FRACTION (F-I) LIBERATED BY HYDRAZINOLYSIS OF α_1-ACID GLYCOPROTEIN

Fraction	*Molar ratio**					*GlcNAc/ Man*	*Weight (μg)*
	Fuc	*Gal*	*Man*	*GlcNAc*	*NeuAc*		
Total monosialyl-oligosaccharides (F-I)	0.25	2	3	3.2	1.1	1.06	2000
F-I-1	0.6	2	3	3	1.1	1	16.2
F-I-2	0.25	2.1	3	3.2	1	1.06	72
F-I-3	0.2	2	3	3.3	0.88	1.1	72
F-I-4	0.3	2.2	3	3.36	0.9	1.12	51
F-I-5	0.3	2.5	3	3.6	0.9	1.2	23
F-I-6	0.24	2.6	3	3.9	1.06	1.3	105
F-I-7	0.31	2.8	3	3.9	0.98	1.3	99
F-I-8	0.36	3	3	4.8	1.1	1.6	90
F-I-9	0.35	2.9	3	4.5	1	1.5	109
F-I-10	0.23	2.8	3	4.4	0.85	1.46	84
F-I-11	0.34	2.8	3	4.6	0.8	1.53	74
F-I-12	0.6	3.2	3	4.3	1	1.43	32
F-I-13	0.66	3.3	3	4	0.9	1.33	27
F-I-14	0.75	3	3	4.2	1.1	1.4	18
Recovery							872.2

* The molar ratio of mannose (Man) was taken as 3.

TABLE IV

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF DISIALYLATED FRACTION (F-II) LIBERATED BY HYDRAZINOLYSIS OF α_1-ACID GLYCOPROTEIN

Fraction	*Molar ratio**					*GlcNAc/ Man*	*Weight (μg)*
	Fuc	*Gal*	*Man*	*GlcNAc*	*NeuAc*		
Total disialyl-oligosaccharides (F-II)	0.25	2.5	3	3.5	2.2	1.16	4000
F-II-1	0.36	2.1	3	3	1.7	1	31
F-II-2	0.37	2.1	3	3.1	1.9	1.03	35
F-II-3	0.18	2.1	3	3.4	2.2	1.13	112
F-II-4	0.21	2.1	3	3.3	2.2	1.1	168
F-II-5	0.2	2.3	3	3.4	2	1.13	73
F-II-6	0.21	2.3	3	3.5	2.2	1.16	238
F-II-7	0.17	2.3	3	3.54	2.1	1.18	300
F-II-8	0.25	2.6	3	3.7	2.1	1.23	146.5
F-II-9	0.3	2.7	3	4	2	1.33	290
F-II-10	0.4	2.8	3	4.25	2.1	1.41	200
F-II-11	0.5	3	3	4.2	2	1.4	160
F-II-12	0.6	3	3	4.5	1.8	1.5	110
F-II-13	0.6	3	3	4.4	1.9	1.46	110
F-II-14	0.7	3	3	4.5	1.6	1.5	70
Recovery							2043

* The molar ratio of mannose (Man) was taken as 3.

viously described[26–28], a monofucosylated biantennary (class AF). Each class shows a high degree of microheterogeneity due to positional isomerism of the sialyl and fucose residues. Methylation analysis (Table V) of oligosaccharides F-I-4 reveals a new structure for the carbohydrate moiety of α_1-acid glycoprotein (Fig. 3): a monosialylated, monofucosylated biantennary oligosaccharide with the trisaccharide Gal $\beta 1 \rightarrow 4$ (Fuc $\alpha_1 \rightarrow 3$) GlcNAc as in the carbohydrate moiety of parotid α-amylase[29] and a sialic acid residue linked to galactose at the C-6 position of the terminal galactose.

The carbohydrate composition of the disialylated oligosaccharides obtained by chromatography on alkylamine shows the presence of biantennary and triantennary oligosaccharides with a high degree of microheterogeneity. Four major fractions were obtained: F-II-6, F-II-7, F-II-9 and F-II-10. Oligosaccharides F-II-6 and F-II-7 have a carbohydrate composition corresponding to a biantennary structure with a small amount of fucose; F-II-9 and F-II-10 possess a carbohydrate composition corresponding to a triantennary oligosaccharide. The difference in chromatographic mobility is due to the positional isomerism of the sialyl and fucose residues, and also to the minor products obtained during hydrazinolysis[30].

Using isocratic conditions with a solvent containing acetonitrile, potassium dihydrogen phosphate and tetraethylpentamine, the trisialylated fraction (F-III) and

TABLE V

METHYLATION ANALYSIS OF MONOSIALYLATED OLIGOSACCHARIDE F-I-4

O-Methyl is abbreviated as OMe, N-acetyl-N-methyl as NAcNMe. NANA = N-acetylneuraminic acid.

Methylated monosaccharides (mol/mol of oligosaccharides)							
2,3,4-tri-OMe-Fuc	*2,3,4,6-tetra-OMe-Gal*	*2,3,4,tri-OMe-Gal*	*3,4,6-tri-OMe-Man*	*2,4-di-OMe-Man*	*3,6-di-OMe-Glc-NAcNMe*	*6-OMe-Glc-NAcNMe*	*4,7,8,9-tetra-OMe-NANA*
0.3	0.81	1.05	1.75	1	2.2	0.5	1

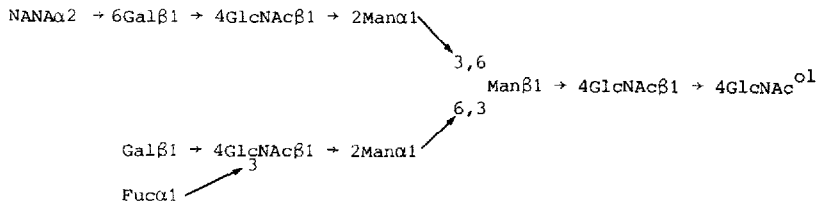

Fig. 3. Proposed structure of oligosaccharide F-I-4.

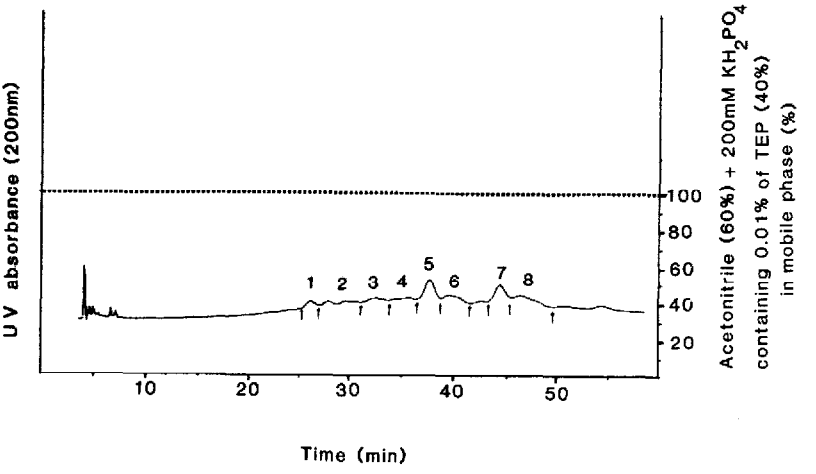

Fig. 4. Analysis of trisialyl-oligosaccharides from the hydrazinolysis of α_1-acid glycoprotein F-III by liquid chromatography on primary amine-bonded silica with acetonitrile–potassium dihydrogen phosphate–tetraethylpentamine as eluent.

the tetrasialylated fraction (F-IV) can be chromatographed on alkylamine-modified silica (Figs. 4 and 5). Eight peaks for the trisialylated fraction and eleven peaks for the tetrasialylated fraction were obtained. The carbohydrate compositions and weights of the oligosaccharides obtained from the trisialylated and tetrasialylated fractions are given in Table VI and VII respectively.

The trisialylated fraction is characterized by classical triantennary oligosaccharides with a high degree of microheterogeneity due to the position of attachment of fucose and sialic acid.

HPLC of the tetrasialylated fraction gives a very interesting pattern of different isomers with four sialic acid residues. Fractions IV-3 to IV-8 show classical tetraantennary oligosaccharides with a high degree of microheterogeneity already mentioned by van Halbeek *et al.*[31] in connection with the position of attachment of fucose on N-acetylglucosamine 7,7′ and 5′, but also due to positional isomerism of the sialyl residues in α 2-3 and α 2-6 to external galactose residues. Fractions IV-9 to IV-11 comprise oligosaccharides with an additional lactosamine residue, as found in human α_1-acid glycoprotein by Yoshima *et al.*[28] (oligosaccharides H 6-11) and in group IV glycopeptides isolated from human asialo α_1-acid glycoprotein by Schmid *et al.*[25]

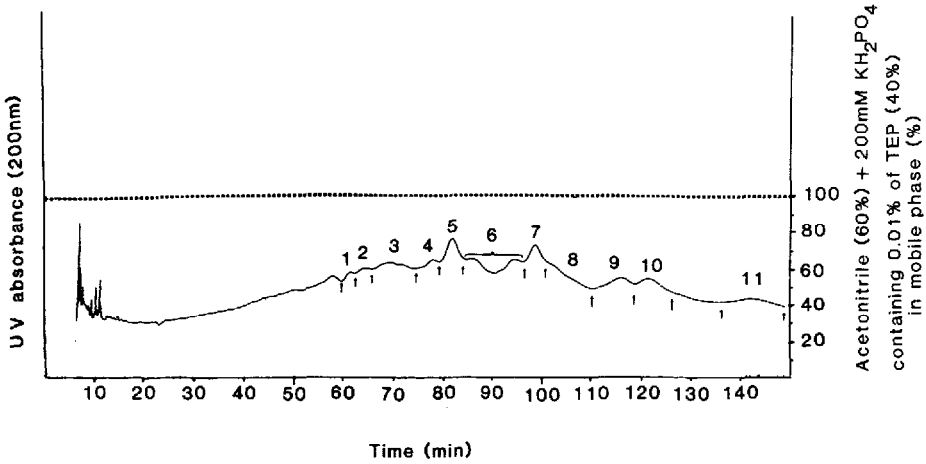

Fig. 5. Analysis of tetrasialyl-oligosaccharides from the hydrazinolysis of α_1-acid glycoprotein F-IV by liquid chromatography on primary amine-bonded silica with acetonitrile–potassium dihydrogen phosphate–tetraethylpentamine as eluent.

TABLE VI

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF TRISIALYLATED FRACTION (F-III) LIBERATED BY HYDRAZINOLYSIS OF α_1-ACID GLYCOPROTEIN

Fraction	*Molar ratio**					*GlcNAc/ Man*	*Weight (μg)*
	Fuc	*Gal*	*Man*	*GlcNAc*	*NeuAc*		
Total trisialyl-oligosaccharides (F-III)	0.6	3.3	3	4.4	3.2	1.46	4000
F-III-1	0.34	2.7	3	4.1	3.2		100
F-III-2	0.36	2.6	3	4.2	3		180
F-III-3	0.4	2.7	3	4.2	3		180
F-III-4	0.44	2.7	3	4.2	3.1		230
F-III-5	0.46	2.9	3	4.3	3.1		170
F-III-6	0.54	2.9	3	4.4	3.1		240
F-III-7	0.65	3.1	3	4.5	3.2		130
F-III-8	0.6	3.3	3	4.4	3.2		300
Recovery							1530

* The molar ratio of mannose (Man) was taken as 3.

(glycopeptides IV-2 and IV-3). As mentioned by Yoshima *et al.*[28], these oligosaccharides are found only in tetra-antennary sugar chains.

These results indicate that it is now possible to analyse high-molecular-weight anionic oligosaccharides by HPLC. The procedure described allows the fractionation

TABLE VII

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF TETRASIALYLATED FRACTION (F-IV) LIBERATED BY HYDRAZINOLYSIS OF α_1-ACID GLYCOPROTEIN

Fraction	*Molar ratio**					*GlcNAc/ Man*	*Weight (μg)*
	Fuc	*Gal*	*Man*	*GlcNAc*	*NeuAc*		
Total tetrasialyl-oligosaccharides (F-IV)	0.7	4.3	3	5.2	3.9	1.73	
F-IV-1							
F-IV-2							
F-IV-3	0.6	4	3	4.8	4	1.6	25
F-IV-4	0.7	3.8	3	4.4	4	1.46	15
F-IV-5	0.55	4.16	3	5.2	4	1.73	72
F-IV-6	0.65	4	3	5	4	1.66	40
F-IV-7	0.65	4.1	3	5.1	4	1.7	102
F-IV-8	0.9	4	3	4.9	4	1.63	40
F-IV-9	0.6	5	3	5.7	4	1.9	12
F-IV-10	0.65	4.64	3	5.81	4	1.93	11
F-IV-11	1	5.3	3	6.5	4	2.16	28.5
Recovery							345.5

* The molar ratio of mannose (Man) was taken as 3.

of isomers of polysialylated N-acetyllactosaminic-type oligosaccharides within a short time. These separations are due to the high concentration of phosphate buffer and to the addition of DAB or tetraethylpentamine in the eluent. With these techniques, the different isomers of sialooligosaccharides obtained by hydrazinolysis of N-acetyllactosaminic-type glycoproteins can be studied.

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