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DETERMINATION OF MONOSACCHARIDES IN GLYCOPROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON 2.1-mm NARROWBORE COLUMNS

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SUMMARY

The monosaccharide composition of glycoproteins was determined by using a Supelcosil LC-18 150 × 2.1 mm narrowbore column under reversed-phase conditions. Methodology was developed for sample hydrolysis, N-acetylation, benzoylation, and cleanup prior to high-performance liquid chromatography. Monosaccharides were identified and quantified at picomole levels. Various glycoproteins were then analyzed, including ribonucleases A and B, Mucin, and fetuin. In addition, the monosaccharide content of Chinese hamster ovary cell-expressed recombinant human renin was determined.

INTRODUCTION

Glycoproteins are found in a wide variety of proteins, including enzymes, immunoglobulins, carriers, hormones, toxins, lectins, and structural proteins. Of the more than 200 known monosaccharides, only 11 have been found in glycoproteins. Of these, seven are common[1]. The monosaccharides occurring most commonly in glycoproteins include glucose, galactose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine, and sialic (N-acetylneuraminic) acid. All but fucose occur in the D-form.

A number of methods have been proposed for analyzing monosaccharides derived from glycoproteins. Resolution by gas–liquid chromatography of trimethylsilylated monosaccharides is a commonly applied methodology[2–8]. However, the instability of the trimethylsilylated derivatives makes this method less than ideal for routine analysis.

Daniel *et al.*[9] and more recently Jentoft[10] have reported procedures for analyzing the carbohydrate composition of glycoproteins by high-performance liquid chromatography (HPLC). These methods include methanolysis[11] and re-N-acetylation, followed by benzoylation, sample cleanup by solid phase extraction[12], and reversed-phase HPLC[10,12].

The intent of this study is to demonstrate the usefulness of 2.1-mm narrowbore columns in a conventional HPLC system for routine microanalysis for monosac-

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charides. Retention times and anomeric area ratios were determined for benzoylated sugar standards. Glycoproteins from a variety of samples, including ribonucleases A and B, mucin, and fetuin, were analyzed for their monosaccharide content. The monosaccharide content of Chinese hamster ovary cell-expressed recombinant human renin was determined with this method.

EXPERIMENTAL

Materials

Supelcosil LC-18, 150 × 2.1 mm columns (Supelco, Bellefonte, PA, U.S.A.) were used for reversed-phase chromatography. Reagents, standards, and other chemicals were obtained from the following sources: benzoic anhydride, dimethylaminopyridine, sugar standards, and glycoprotein standards from Sigma (St. Louis, MO, U.S.A.), reagent-grade pyridine, acetic anhydride, iodine, and magnesium turnings from Mallinckrodt (Paris, KY, U.S.A.), acetyl chloride from Aldrich (Milwaukee, WI, U.S.A.), and hexamethyldisilazane from Petrarch Systems (Levittown, PA, U.S.A.). Chinese hamster ovary cell-expressed recombinant human renin was obtained through the courtesy of Dr. Roger Poorman (The Upjohn Co., Kalamazoo, MI, U.S.A.) from a previously described preparation[13]. Reacti-Vials (1.0 ml) were obtained from Pierce (Rockford, IL, U.S.A.). Supelclean LC-18 solid-phase extraction tubes (3 ml) were provided by Supelco.

Dry methanol was prepared by adding 2.5 g magnesium turnings and 0.1 g iodine to 500 ml HPLC-grade methanol, refluxing for 1 h, and purifying the product by distillation in an all-glass still. Methanolic HCl (1.0 *M*) was prepared by adding acetyl chloride to dried methanol that had been cooled on dry ice for several minutes*.

The perbenzoylating mixture benzoic anhydride–dimethylaminopyridine–pyridine (10:5:85, w/w/v) was freshly prepared and not used if more than 1/2 day old. A mixture of *tert.*-butanol and methanol (4:1, v/v) was used in the evaporation. Glassware was silanized with a solution of 10% (v/v) hexamethyldisilazane in toluene. All other solvents were of HPLC grade.

Methods

Benzoylated sugars were prepared by the Jentoft method[10], and the resulting samples were freed of reaction byproducts by using a solid-phase extraction procedure similar to that of Daniel *et al.*[9].

Derivatizations

Derivatizations were carried out on the oligosaccharide, glycoprotein, or standard sugar samples (0.2–1.0 mg/ml) in a 1-ml Reacti-Vial, first by lyophilizing or drying the sample under nitrogen. The dried sample was then hydrolyzed by adding 0.2 ml of 1 *M* methanolic HCl, sealing the vial with a PTFE-lined screw cap and heating at 65°C for 16 h. The sample was then cooled to room temperature and re-N-acetylated. The sample was resuspended in 0.1 ml *tert.*-butanol–methanol (4:1, v/v) and dried again. Re-N-acetylation of the hexosamines was performed by dis-

* This addition must be carried out in a dropwise fashion, because the reaction of acetyl chloride with any protic solvent is violently exothermic.

solving the sample in 0.1 ml dry methanol, adding 40 μl pyridine, followed by 40 μl acetic anhydride, and incubating for 1 h at room temperature. Excess pyridine was removed by drying the sample, dissolving in 40 μl toluene, and drying again. The sample was hydrolyzed again with 0.2 ml of 0.1 *M* methanolic HCl at 65°C for 30 min. The sample was then dried, redissolved in dry methanol, transferred to a silanized Reacti-vial, and dried again. Perbenzoylation was carried out by adding 0.1 ml fresh benzoic anhydride–dimethylaminopyridine–pyridine (10:5:85, v/w/v) to the sample and incubating at room temperature for 16 h. The benzoylation was stopped by adding 0.9 ml water to the vial and incubating at room temperature for 30 min. If the sample is not miscible, sonicate for 3–6 min before incubation.

Sample cleanup

Sample cleanup was carried out by solid-phase extraction. A 3-ml Supelclean LC-18 solid-phase extraction tube was conditioned with 3.0 ml acetonitrile, followed by 5.0 ml distilled water. The aqueous, derivatized sample was added and the tube washed with 3 × 2 ml of water to remove byproducts. The sample was removed from the tube in 3 × 2 ml acetonitrile washings. The acetonitrile fractions were pooled, dried, and redissolved in 1.0 ml initial HPLC mobile phase (acetonitrile–water, 35:65) for analysis.

Chromatography was performed on a Varian 5000 HPLC system, using a Varian 9090 auto sampler (Varian, Walnut Creek, CA, U.S.A.), a Kratos 773 UV detector with a 2.4-μl cell (Kratos Analytical, Ramsey, NJ, U.S.A.), and a Varian 401 Vista data system. Monosaccharide standards were prepared and chromatographed individually in order to determine retention times and anomer ratios. Chromatographic conditions were as follows: column, 150 × 2.1 mm Supelcosil LCC-18; mobile phase, acetonitrile–water (35:65) (A) and (90:10) (B); gradient conditions, 65 min linear gradient from 100% A to 100% B, held at 100% B for 10 min, followed by immediate reequilibration to 100% A for 15 min before the next injection; flow-rate, 0.6 ml/min; pressure, 148–155 bar; chart speed, 0.2 cm/min; temperature, ambient; detection, 230 nm UV; injection volume, 2.0 μl.

TABLE I

RETENTION TIMES (min) OF BENZOYLATED METHYL GLYCOSIDE STANDARDS ON A SUPELCOSIL LC-18, 150 × 2.1 mm COLUMN (5 μm PACKING)

Methyl glycoside	*Anomer*			
	1	*2*	*3*	*4*
Fucose	28.7	31.4	35.6	–
Glucose	36.8	39.9	–	–
N-Acetylgalactosamine	16.3	19.6	21.6	30.0
Galactosamine	16.6	19.9	22.0	30.2
Mannose	34.5	39.1	–	–
Galactose	36.9	40.7	54.3	57.5
N-Acetylglucosamine	17.5	20.7	–	–
Sialic acid	31.7	–	–	–

TABLE II

ANOMER RATIOS OF BENZOYLATED METHYL GLYCOSIDES

Anomer ratio is defined as the area count for the anomer divided by the total sugar area counts, based on the retention times in Table I.

Methyl glycoside	*Anomer*			
	1	*2*	*3*	*4*
Fucose	0.27	0.57	0.15	–
Glucose	0.27	0.73	–	–
N-Acetylgalactosamine	0.10	0.70	0.15	0.03
Galactosamine	0.29	0.35	0.25	0.10
Mannose	0.07	0.93	–	–
Galactose	0.25	0.69	0.02	0.04
N-Acetylglucosamine	0.23	0.77	–	–

RESULTS AND DISCUSSION

A sugar cleaved from a glycoprotein can exist in as many as four anomeric forms, depending upon the specific stereochemistry of the sugar. Retention times for the most commonly occurring benzoylated methyl glycoside anomers are summarized in Table I. Table II summarizes the measured anomer ratios for seven of these derivatized sugars. In combination, relative retentions and anomer ratios can be used in identifying these or other monosaccharides from glycoproteins.

A major advantage of this procedure over gas chromatographic analyses is that the molar response factors do not vary, because the response depends only on

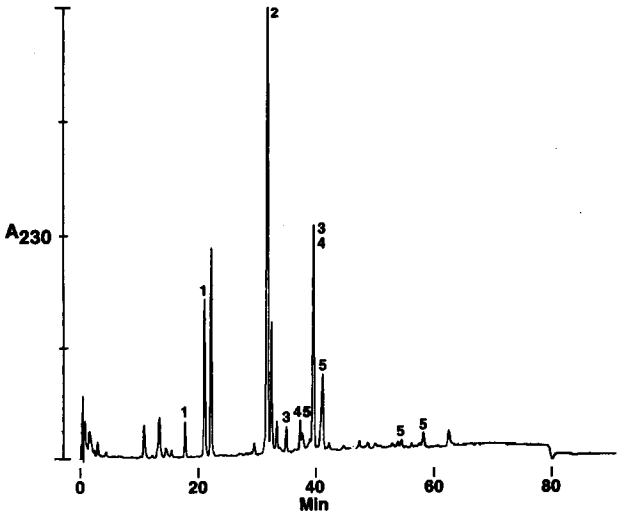

Fig. 1. Chromatogram of a five-component standard monosaccharide mixture. Experimental conditions described in Materials and methods. Peak identification: 1 = N-acetylglucosamine; 2 = sialic acid; 3 = mannose; 4 = glucose; 5 = galactose.

the number of benzoyl groups. Molar reactions of a given monosaccharide can be calculated by summing the areas of all anomers of that monosaccharide. The anomer ratios can be used in identifying the original sugar.

Fig. 1 shows the chromatographic separation of a five-component standard test mixture, containing N-acetylglucosamine, sialic acid, mannose, glucose, and galactose. The retention times were verified by adding monosaccharide standards to the sample and were used in identifying the components of the glycoproteins. Sialic acid shows a second minor peak (Fig. 1) with a retention time of 32.5 min for some of the high concentration standards evaluated. Since this second sialic acid peak appeared to be sample dependent and, since it was not verified in the real samples, it was not used for the composition determinations.

The monosaccharide content was determined for ribonucleases A and B, two types of mucin, and fetuin. Fig. 2 shows the differences between the glycosylated and non-glycosylated forms of ribonuclease, *i.e.* ribonuclease B and ribonuclease A. Both glycoproteins have the same amino acid composition, but ribonuclease B contains eight carbohydrate units per molecule, namely six residues of mannose and two residues of N-acetylglucosamine[14]. In Fig. 2, peaks corresponding to N-acetylglucosamine and mannose standards are easily detected in the chromatograms for ribonuclease B, despite the small size (2–5 pmol) of the sample. A ratio of 6.0 mannose to 1.75 N-acetylglucosamine residues was found for the ribonuclease B sample, compared to the 6.0:2.0 theoretical ratio.

Measured values for the monosaccharide content of a particular glycoprotein

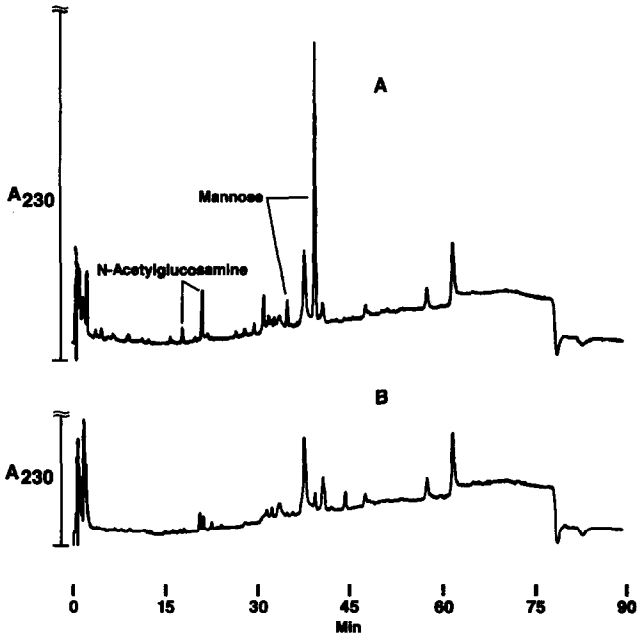

Fig. 2. Chromatograms of the monosaccharides in bovine pancreatic ribonucleases B and A. (A) Ribonuclease B (2.62 pmol on-column) contained N-acetylglucosamine (5.24 pmol) and mannose (15.72 pmol). (B) Ribonuclease A (35.3 pmol on-column) contained only trace amounts of N-acetylglucosamine or mannose.

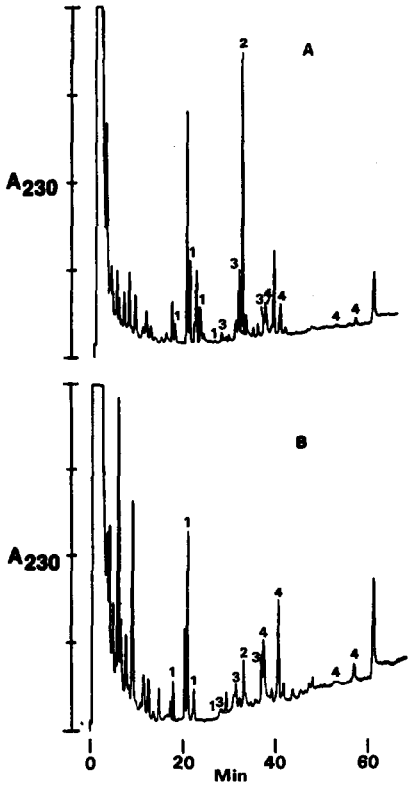

Fig. 3. Chromatograms of the monosaccharides in mucin. (Data summarized in Table III.) Peak identification: 1 = N-acetylgalactosamine; 2 = sialic acid; 3 = fucose; 4 = galactose. (A) Mucin type I (bovine submaxiallary gland), (B) mucin type II (porcine stomach).

can reflect the source, purity, condition, method of preparation, and stability of the sample. An example of source differences is shown in Fig. 3, in which the profiles of mucin type I (from bovine submaxilliary glands) and mucin type II (from porcine stomach) are compared. Some peak overlap is seen, as shown in Fig. 3 when profiling is carried out with biologic samples. As an example, Jentoft[10] showed some overlapping when anomers of fucose and galactose are present. Component identifications were verified by adding monosaccharide standards to the samples.

Table III compares the monosaccharide content of porcine submaxillary mucin proteins from reports by Jentoft[10] and De Salegui *et al.*[15] with the values for bovine submaxillary mucin and porcine stomach mucin determined in this study. Our results for both bovine and porcine mucin are compatible with those for the commercial type I mucin, with the exception of the sialic acid content.

Fig. 4 shows a chromatographic profile of the monosaccharides bound to fetal calf fetuin. Our data and literature data[10,16] are summarized in Table III and agree very well for four of the five sugars (galactose, sialic acid, mannose, and N-acetyl glucosamine) in fetuin. This sample contained about twice as much N-acetylgalactosamine as reported elsewhere[15]. Jentoft also reported considerable variation in

TABLE III

MONOSACCHARIDE CONTENT OF MUCIN AND FETUIN GLYCOPROTEIN SAMPLES

All data are molar ratios, normalized to galactose.

Sample	*Monosaccharide*	*Reported values*		*This study*	
		*Ref. 10, porcine**	*Ref. 15, porcine**	*Mucin I, bovine***	*Mucin II, porcine****
Mucin	Galactose	1	1	1	1
	Glucose	0	0	0	0
	Sialic acid	0.78	0.85	4.2	0.19
	N-Acetylglucosamine	0	0	0	0
	N-Acetylgalactosamine	2.01	2.15	2.11	1.51
	Fucose	0.88	0.94	1.2	0.74
	Mannose	0	0	0	0
		Ref. 10, fetal calf serum§	*Ref. 16, fetal calf serum**	*This study; fetal calf serum*§§	
Fetuin	Galactose	1	1	1	
	Glucose	0	0	0	
	Sialic acid	0.87	1.08	1.03	
	N-Acetylglucosamine	1.19	1.25	1.33	
	N-Acetylgalactosamine	0.22	0.25	0.53	
	Fucose	0	0	0	
	Mannose	0.82	0.75	0.82	

* Fresh preparation.
** Sigma M4503 from bovine submaxillary glands, *ca.* 5% bound sialic acid (see Fig. 3A).
*** Sigma M2378 from porcine stomach, *ca.* 1% bound sialic acid (see Fig. 3B).
§ Sigma preparation.
§§ Sigma F2379 from lyophilized fetal calf serum, *ca.* 0.2% free sialic acid (see Fig. 4).

carbohydrate composition in mucin and fetuin preparations from different animals[10].

The methodology described in this study can be used not only to investigate interspecies differences in glycoprotein composition, but also to determine purity, microheterogeneity, effects of storage, differences in sample preparation methods, and hepatic uptake.

Fig. 5 shows the chromatographic identification of four monosaccharides from Chinese hamster ovary cell-expressed recombinant human renin[13]. N-Acetylglucosamine, sialic acid, mannose, and galactose were present in this sample in a 6:4:6:3 molar ratio. Fig. 5 also shows that four peaks in the sample can be attributed to benzoylated buffer components. This conclusion was confirmed by standard additions.

The methodology described here for monitoring the monosaccharide composition of glycoproteins offers several advantages over the classical gas chromatographic methods. The sensitivity of the method is excellent; less than 1 nmol of a benzoylated sugar can be detected by using the narrowbore column in a standard LC system. Derivatization and sample cleanup methods were conveniently carried out

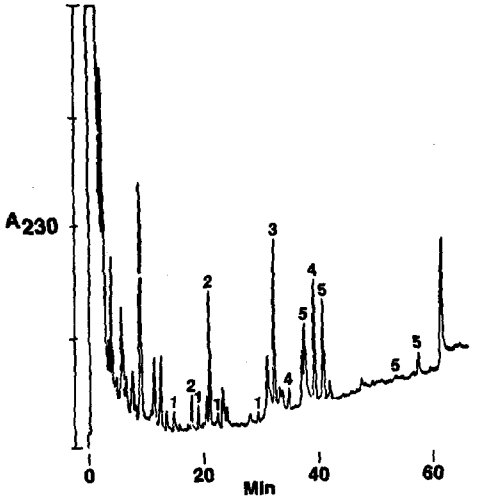

Fig. 4. Chromatogram of the monosaccharides in fetuin. Peak identification: 1 = N-acetylgalactosamine; 2 = N-acetylglucosamine; 3 = sialic acid; 4 = mannose; 5 = galactose.

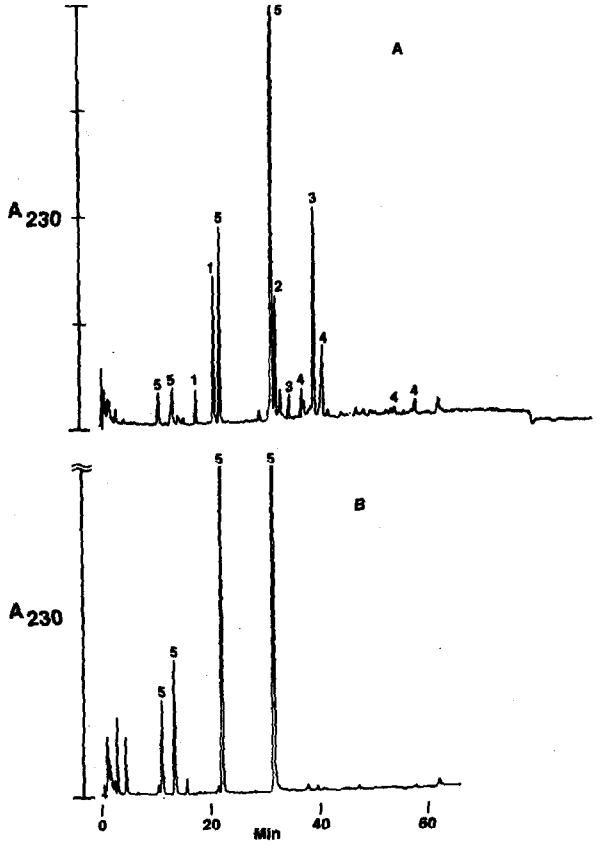

Fig. 5. Chromatograms of the monosaccharides in renin. Peak identification: 1 = N-acetylglucosamine; 2 = sialic acid; 3 = mannose; 4 = galactose; 5 = distinctive benzoylated buffer peaks. (A) Renin, (B) benzoylated buffer blank [0.1 *M* sodium acetate (pH 4.3 with glacial acetic acid), pH 7.0 with a saturated Tris solution].

by using a 0.1–1.0 mg/ml solution of either protein or standard. Limiting factors are sample preparation and work-up manipulations.

Another limiting factor in carbohydrate analyses in general has been the amount of glycoprotein required. The work presented here shows that a HPLC technique can be very sensitive. For the ribonuclease B sample used to obtain Fig. 2, the sugar content was only 10.4% of the total molecular weight. Thus, a 2.6-pmol (38.5 ng) sample contained only 4 ng of monosaccharide, mannose and N-acetylglucosamine, in a 6:2 molar ratio. Although only 2.8 ng of mannose and 1.2 ng of N-acetylglucosamine were present in the 38.5 ng sample, the peaks could easily be detected with this method. If necessary, the lower limits of detection can be enhanced by using a microbore LC system with a reduced detector cell volume, rather than a conventional LC system. A microbore LC system will enhance the level of detection about four-fold[10]. Although it takes *ca.* three days to prepare the benzoylated derivatives, the resulting samples are stable for several months. Consequently, the analysis can be repeated or delayed without loss of information. Second, the fractions corresponding to chromatographic peaks can be collected for further characterization (*e.g.*, by mass spectroscopy).

CONCLUSIONS

Benzolated monosaccharide samples produced by the Jentoft method can be readily analyzed by using a 150 × 2.1 mm column in a conventional HPLC system. Sugar type and relative content can be established at picomole detection levels. Measurements of the monosaccharide content of ribonuclease, mucins, fetuin, and Chinese hamster cell-expressed recombinant human renin demonstrate the versatility of this method.

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