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MEASUREMENT OF NEUTRAL SUGARS IN GLYCOPROTEINS AS DANSYL DERIVATIVES BY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Automated high-performance liquid chromatography was used to analyse dansylhydrazine derivatives of neutral sugars in unfractionated acid hydrolysates of four well-characterized glycoproteins: fetuin, ovalbumin, alpha-1-acid glycoprotein and bovine submaxillary mucin. After a simple single-step derivatization at 65°C the sugar derivatives in protein hydrolysates chromatographed as single peaks on reversed-phase C_{18} columns. The isocratic solvent consisted of 20% (v/v) aqueous acetonitrile containing 0.01 *M* formic acid, 0.04 *M* acetic acid and 0.001 *M* triethylamine. The triethylamine significantly increases the sugar peak height at 254 nm. Repeated automatic sample injection without deterioration of column performance or interference from dansyl hydrazine is not possible with published methods, but was achieved by cleaning the column between each analysis with a solvent of 20% (v/v) acetonitrile and 80% (v/v) methanol. Hydrolysis with 2 *M* trifluoroacetic acid is superior to 2 *M* hydrochloric acid for both sugar recovery and convenience but must continue for 6–8 h at 105°C to ensure complete sugar release. We confirmed that mannose is present in most preparations of human high-molecular-weight salivary glycoproteins, and also examined purified bovine skin proteodermatan sulphate. *p*-Nitrophenylhydrazine derivatives of neutral sugars are readily produced, but do not chromatograph as successfully as the dansyl derivatives while phenylhydrazine derivatives are not easily produced at 65°C. Further development of the method should be possible by producing other hydrazine derivatives of neutral sugars.

INTRODUCTION

Measurement of the neutral sugars of glycoproteins is of importance in the purification of glycoproteins, in providing structural information and in helping to understand glycoprotein properties such as immunogenicity. The principal methods in current use for routine detection and quantitation of neutral sugars are thin-layer chromatography (TLC)[1], gas–liquid chromatography (GLC) of volatile sugar derivatives[2] and liquid chromatography of neutral sugars and their derivatives[3]. Various methods have been employed to selectively detect and quantitate neutral sugars using

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high-performance liquid chromatography (HPLC) either by pre-column derivatization of sugars[4–6] or by post-column reactions[7,8]. Underivatized neutral sugars cannot be detected selectively and require pre-column separation from contaminating substances. Methods for amino sugars and sialic acids are not readily integrated with analysis of neutral sugars without complicating the overall analysis since they pose problems of appropriate hydrolysis, sample purification and speed of chromatography[7–11].

Alpenfels[4] and Mopper and Johnson[5] developed an HPLC method on C_{18} columns for highly sensitive quantitation of dansyl derivatives of pure neutral sugars. Derivatization with dansylhydrazine exhibits specificity for neutral aldoses[1,4]. Our objective was to use chromatography of dansyl derivatives of neutral sugars for automated HPLC analysis of neutral sugars in parallel with automated amino acid analysis for characterization of fractions generated during glycoprotein purification. We encountered difficulties with the published methods for HPLC of dansylated neutral sugars which made automated HPLC impossible without modification. Development of a routine automated method also required assessment of the stability of the dansyl derivatives, choice of an internal sugar standard, identification of optimum hydrolysis conditions and investigation of the effect of protein hydrolysis on neutral sugar analysis. We used both the *p*-nitrophenyl- and the phenylhydrazine derivatives of neutral sugars to investigate the effect of derivative type on chromatographic properties and were able to indicate approaches for future development of the method.

MATERIALS AND METHODS

All chemicals were of analytical grade. There was no notable variation in solvents from different sources, provided that the acetonitrile and methanol were certified HPLC grade with a stated UV cut-off of 190 nm. Dansylhydrazine, phenylhydrazine, and *p*-nitrophenylhydrazine were obtained from Sigma (St. Louis, MO, U.S.A.) as were pure standard sugars. The reference glycoproteins ovalbumin (lot 108C-8045), calf serum fetuin Type III (lot 91F-9535), alpha-1-acid glycoprotein (lot 32F-9330) and bovine submaxillary mucin Type I (lot 113F-0461) were also obtained from Sigma. Human salivary glycoproteins insoluble in the presence of cetyltrimethylammonium bromide were fractionated by size-exclusion chromatography on Merck Fractogel[12]. Purified bovine skin proteodermatan sulfate[13] was also analysed.

Equipment and columns were as follows: either a Gilson HPLC controller and pumps (Gilson, U.S.A.) or an Altex HPLC controller and pumps (Beckman, Canada); either a simple loop injector with a 20-μl loop (Rheodyne, U.S.A.) or a Waters WISP automatic multiple sample injector (Waters Scientific, Canada); Alltech C_{18} reversed-phase columns (600 RPB, Alltech, U.S.A.) 25 × 4.6 mm O.D., 10 μm particle size; a variety of HPLC UV detectors, including Altex (Beckman) and Pharmacia (Pharmacia, Canada) HPLC models measuring at 254 mm with 8-μl flow cells; a dual-pen chart recorder and a Beckman Model 126 integrator.

Glycoproteins were hydrolysed in sealed vials under nitrogen at 105°C with either 2 *M* trifluoroacetic acid (TFA) or 2 *M* hydrochloric acid. An internal sugar standard was added immediately on opening the vials after hydrolysis. The TFA was removed by lyophilization. Hydrochloric acid was removed by lyophilization either

directly or after neutralization with the bicarbonate form of Dowex 1X8 ion-exchange resin. The effect of protein hydrolysis on sugar recovery and detection was tested by mixing 1 mg of bovine serum albumin (Sigma) with 500 n*M* of each of the standard sugars followed by time-course hydrolysis with 2 *M* TFA or 2 *M* hydrochloric acid at 105°C.

Sugar standards and glycoprotein hydrolysates were derivatized by mixing the sample dissolved in 250 μl of distilled water with 225 μl of 1% (w/v) dansylhydrazine dissolved in ethanol and 45 μl of 10% (w/v) aqueous trichloroacetic acid, heating at 65°C for 20 min followed by a partial purification on Sep-Pak C_{18} cartridges using a vacuum manifold (Waters, U.S.A.)[5]. J. T. Baker octadecyl C_{18} cartridges (J. T. Baker, U.S.A.) were also used. This partial purification prevents deterioration of column performance but only removes the trichloroacetic acid used during derivatization and other hydrophilic substances since most of the unchanged dansylhydrazine cannot be separated in this step without excessive loss of neutral sugar derivatives. The cartridges are used 10 times with regeneration[5]. We found column switching for on-line sample cleanup[5] impractical with our existing equipment for automated HPLC. Samples were next lyophilized under good vacuum (less than 10 μmHg) with a condenser at below −45°C, open air ballast on the vacuum pump and constant scavenging of the exhaust to remove acetonitrile vapour. The dried samples were stored in the dark at −20°C for analysis at any convenient time. Sugar standards were dissolved in 2 ml of 20% (v/v) aqueous acetonitrile to give 5 n*M* of each sugar in 20 μl of solution injected onto the column. Glycoprotein hydrolysates were dissolved in 200 μl of 20% (v/v) acetonitrile with 20 μl injected directly onto the HPLC column.

The chromatographic conditions for separation of sugar derivatives are isocratic with a flow-rate of 1 ml/min of solvent consisting of 20% (v/v) aqueous acetonitrile containing 0.01 *M* formic acid, 0.04 *M* acetic acid and 0.001 *M* triethylamine. The column was cleaned for 15 min between sample injections by switching at a constant flow-rate of 1 ml/min to a solvent consisting of 20% (v/v) acetonitrile and 80% (v/v) methanol for 5 min, and then back to the running solvent. Although an HPLC gradient programmer makes this change of solvents automatic, because the basic separation uses an isocratic solvent the entire method can be performed with a single pump and manual solvent switching.

TLC of derivatives was performed on reversed-phase octadecyl C_{18} silica plates (HETLC-RPS, Analtech, U.S.A.) using ascending chromatography with 20% (v/v) aqueous acetonitrile as the mobile phase. Linear regression was employed[14].

RESULTS AND DISCUSSION

Chromatographic conditions

Triethylamine (0.001 *M*) in the running solvent had no effect on sugar separation[5], but for the different sugars it increased derivative peak heights from two to five times when measured by UV absorption at 254 nm. The dansyl sugar derivatives chromatograph between dansyl hydroxide, which is bound only weakly to the column, and dansylhydrazine, which moves slowly down the column (Fig. 1). This separation is readily confirmed by TLC on reversed-phase C_{18} plates. For successful automated chromatography of dansyl sugar derivtives the column must be non-re-

tentive for dansyl hydroxide and adequately retentive for dansylhydrazine during each analysis. Without column cleaning, repeated injections overload the column capacity for dansylhydrazine, which then elutes in the position of the neutral sugar derivatives after every sample injection and prevents their measurement. This is the major problem preventing application of the published methods to automated analysis. Acetonitrile alone[4] was inadequate to clean the column and restore column capacity for dansylhydrazine. Waiting for dansylhydrazine to elute at its own rate[5] not only increases time per analysis unnecessarily, but does not adequately restore column capacity for dansylhydrazine during subsequent injections. We found a mixture of 20% (v/v) acetonitrile and 80% (v/v) methanol accomplished the necessary column cleaning. Triethylamine and acid were not included in the cleaning solvent because this leads to degradation of column performance. With programming and automatic injection it is possible to analyse one sample per 45 min, and up to fourteen samples have been injected in succession. HPLC of dansyl derivatives of neutral sugars on other column types requires solvent changes to separate all of the sugar derivatives[6] and is therefore not as convenient as the isocratic method.

A single reversed-phase C_{18} column can be used for analyses of different types of glycoprotein carbohydrate component, since dansyl chloride derivatives of amino sugars can be chromatographed on a C_{18} column under almost the same conditions[15] as neutral sugar derivatives.

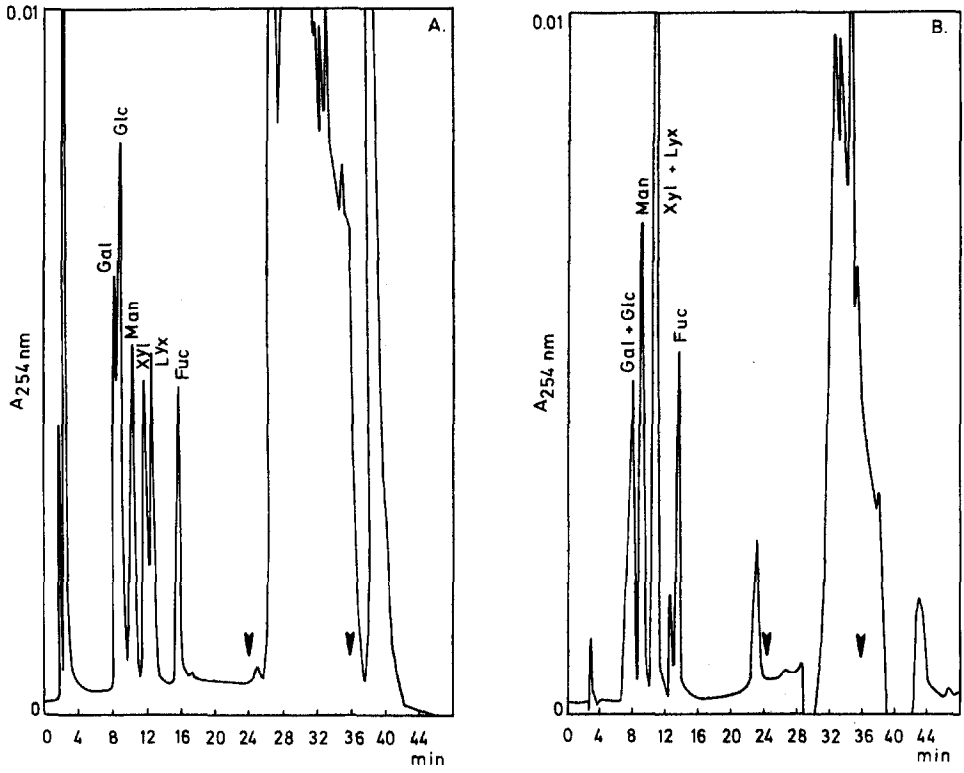

Fig. 1.

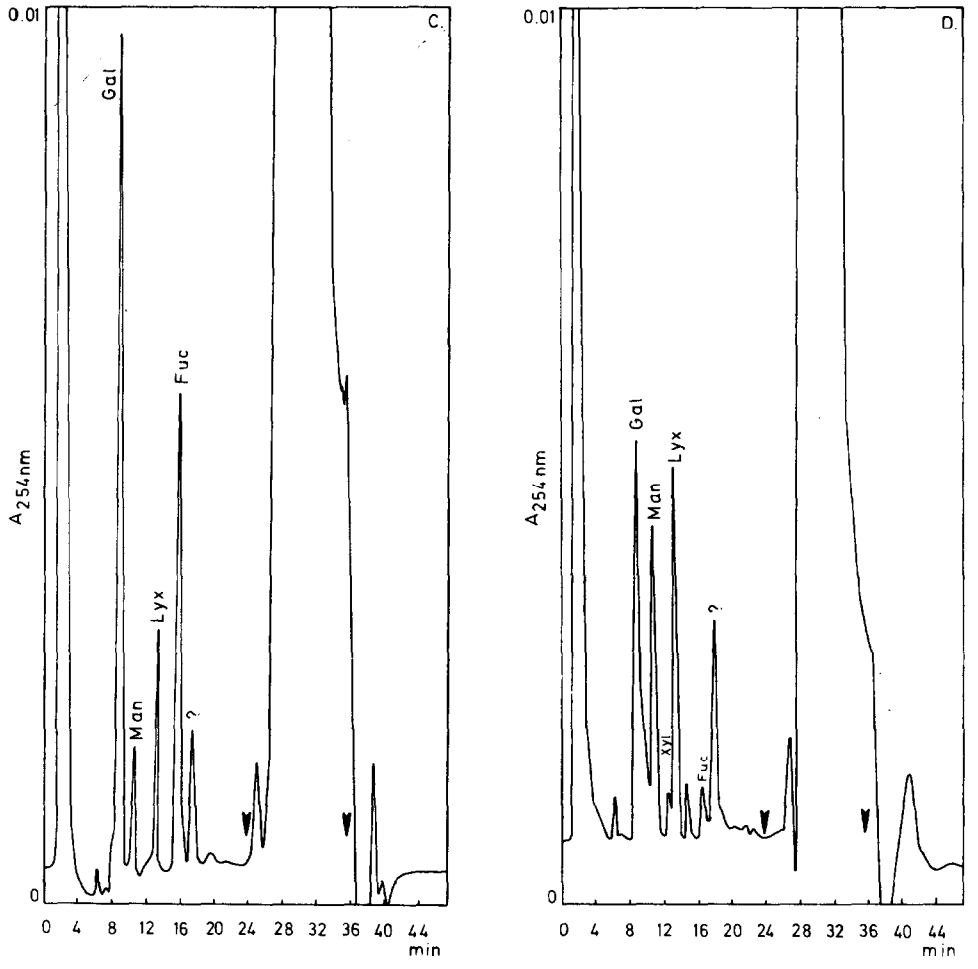

Fig. 1. HPLC on a C_{18} column of (A) dansylhydrazine derivatives and (B) *p*-nitrophenylhydrazine derivatives of neutral sugars; 5 n*M* of each sugar standard injected in 20 μl. Isocratic separating solvent followed by cleaning solvent at 24 min (first arrow) with a change back to separation solvent (second arrow). Solvent in B contained 15% (v/v) acetonitrile instead of 20% (v/v) acetonitrile. (C) Human salivary glycoproteins, 280K MW fraction; (D) bovine skin proteodermatan sulphate. Dansyl derivtives in C and D with chromatography as in A, both proteins hydrolysed in 2 *M* TFA at 105°C for 6 h; lyxose added after hydrolysis of proteins.

Internal standard and choice of derivative type

The current method will not adequately separate dansyl derivatives of fucose and rhamnose, thereby preventing use of rhamnose as an internal standard. Lyxose and xylose derivatives can be separated, so lyxose can be used as an internal sugar standard. An amount of lyxose was added to protein hydrolysates to give final concentration of 5 n*M* per 20 μl of sample injected.

Automatic sample injection over 14 h enabled us to evaluate the stability of dansylhydrazine derivatives of neutral sugars at 20°C. There is an appreciable loss of derivatives over this time, which differs for the various sugars but appears linear

TABLE I

STABILITY OF DANSYL DERIVATIVES OF NEUTRAL SUGARS AT 20°C

Dansyl derivative 5 nM	*Linear regression**					*Loss per hour (%)*
	Intercept peak height (mm)	*Slope height (h)*	*n*	*r*	*p*	
Galactose	155.69	−4.39	15	−0.82	<0.001	2.8
Glucose	179.11	−2.01	15	−0.72	<0.01	1.1
Mannose	120.43	−3.25	15	−0.85	<0.001	2.7
Xylose	119.69	−4.45	15	−0.83	<0.001	3.7
Lyxose	140.79	−9.07	15	−0.87	<0.001	6.4
Fucose	106.72	−2.90	15	−0.84	<0.001	2.7

* n = Number of points per regression; r = correlation coefficient; p = significance value.

with time, thereby allowing calculation of correction factors (Table I). It is not apparent why the lyxose derivative should suffer the greatest deterioration. Our usual practice is to analyse batches of six to eight samples with replicate standards. For manual injection, storage of samples at 4°C minimizes derivative loss.

Examination of other types of sugar derivatives indicated poor derivatization with phenylhydrazine and good derivatization with *p*-nitrophenylhydrazine. The latter derivatives of neutral sugars were partially separated using a running solvent consisting of 15% (v/v) aqueous acetonitrile containing 0.01 *M* formic acid, 0.04 *M* acetic acid and 0.001 *M* triethylamine. The derivatives were detected with approximately the same sensitivity as dansyl derivatives at 254 nm (Fig. 1). We were unable to separate the galactose and glucose peaks or the xylose and lyxose peaks with our standard system. Protein hydrolysates contained a number of unknown peaks when *p*-nitrophenylhydrazine derivatives were made, and so these derivatives were not used for routine analysis.

The elution order of the *p*-nitrophenylhydrazine sugar derivatives was the same as that of the dansyl derivatives (Fig. 1). The order of the derivatives of galactose, glucose, and mannose is repeated by the order of their analogues arabinose, xylose, and lyxose, and again by the galactose and mannose analogues fucose and rhamnose (Fig. 1 and ref. 4). Separation order appears to be affected (a) by the relative orientation of hydroxyl groups on sugar carbons 1 to 4 and (b) by the group attached to sugar carbon 5. While the sugar portion of the derivatives affects elution order, the aromatic portion of the derivative influences the resolving power of the column, as shown by the loss of resolution with the *p*-nitrophenylhydrazine sugar derivatives. It may be possible to produce a derivative with greater resolving power than the dansyl group, thereby permitting adequate separation of fucose and rhamnose derivatives.

Hydrolysis conditions

TFA at 2 *M* and 105°C has previously been used to release neutral sugars from glycoproteins[8,16,17]. Like Honda[7,17] we found 6–10 h at 105°C with 2 *M* TFA the minimum time to give maximum release of sugar from some glycoproteins, such as

proteodermatan sulphate, salivary glycoproteins and alpha-1-acid glycoprotein. From other glycoproteins, such as ovalbumin and fetuin, we found that sugar release with 2 *M* TFA was rapid (4 h) at 105°C. This suggests a minimum hydrolysis time with TFA of 8 h for an unknown glycoprotein and ideally a 6–10 h time-course hydrolysis. The higher temperature (145°C) and 4 *M* TFA concentration used by Neeser and Schweizer[9], as well as the protein analysed, may account for the more rapid release of sugars they observed.

Hydrolysis with 2 *N* hydrochloric acid at 105°C results in a rapid loss of sugars that is greatly accentuated if the acid is not neutralized prior to freeze-drying, but even neutralization of hydrochloric acid prior to freeze-drying does not eliminate all sugar loss (Table II). TFA, which is easily removed by simple lyophilization without need for additional sample treatment, provided the highest sugar recoveries after hydrolysis. An alternative to hydrolysis is methanolysis, but this produces two anomeric peaks for each neutral sugar both in GLC and in HPLC[15,18]. Oxime derivatives of neutral sugars also produce two anomeric peaks during GLC[9]. The single sugar peaks produced by hydrolysis and dansylation greatly simplify the calculation of results and permit automatic integration of the results[4].

Addition of neutral sugar standards during hydrolysis of a non-glycosylated protein, bovine serum albumin, resulted in no unknown peaks in the sugar chromatogram and provided no interference with the method. With TFA hydrolysis, time-course values for five sugar standards extrapolated (linear regression) to within 3.9 +/− 1.4% (S.E.M., $n = 5$) of added amounts. The overall rate of destruction (slope of regression) of added sugar standards was 4.6 +/− 0.8% (S.E.M., $n = 5$) per hour when present during 2 *M* TFA hydrolysis of bovine serum albumin at 105°C. Sugar losses were less during hydrolysis of an actual glycoprotein (Table II). These rates of sugar loss are comparable with the 1.0–4.0% loss per hour encountered by others using 2 *M* TFA as hydrolytic agent[17].

Analysis of reference glycoproteins

Time-course hydrolysis using 2 *M* TFA gave results for neutral sugar compositions of the well-characterized glycoproteins fetuin, ovalbumin and alpha-1-acid glycoprotein closely comparable with published values obtained with different

TABLE II

EFFECT OF HYDROLYSIS AGENT AND SAMPLE PROCESSING ON NEUTRAL SUGARS

Time course hydrolysis: 4, 6, 8 h at 105°C.

Fetuin	*Hydrolysis 2 M HCl lyophilized*	*Hydrolysis 2 M HCl neutralized lyophilized*	*Hydrolysis 2 M TFA lyophilized*	*Published values refs. 1, 3, 7, 8*
Extrapolated values (linear regression intercept) g sugar/100 g sample				
Galactose	1.93	2.98	4.17	(3.5–4.6)
Mannose	1.52	2.23	2.40	(2.3–3.1)
Sugar losses per hour of hydrolysis (slope of regression)				
Mean value	2.0%	1.5%	2.7%	

methods (Table III). These values suggest an absence of interfering substances in unfractionated protein hydrolysates. There was reasonable agreement with published values for the less well-characterized bovine submaxillary mucin (Table III). Some glycoproteins produce an unknown peak following the fucose derivative (Fig. 1). It is a mark of excessive column deterioration when this unknown peak and the fucose peak can no longer be separated clearly. Mixtures of standard sugars were derivatized each time a glycoprotein sample was analysed. It is possible to analyse as little as 125 μg of glycoprotein, but the derivatives must be taken up in only 50 μl of solvent. We can routinely detect and quantitate 200–300 pmol of neutral sugars per injection of 20 μl of protein hydrolysate. This is almost as sensitive as another recently reported method[7], but with much lower consumption of acetonitrile and much simpler equipment.

Analysis of unknown glycoproteins

The method was applied to highly purified bovine skin proteodermatan sulphate without apparent interference from the glycosaminoglycan component of the molecule, since xylose of the glycosaminoglycan linkage region was detected and measured (Fig. 1D, Table III). We have not analysed cartilage-type proteoglycans.

TABLE III

NEUTRAL SUGAR COMPOSITIONS OF GLYCOPROTEINS

Values obtained from time-course hydrolysis (linear regression intercept), expressed as grams of sugar per 100 grams of sample*.

	*Gal***	*Man*	*Xyl*	*Fuc*	*Glc*	*Ref.*
Calf serum fetuin	4.17 (3.5–4.6)	2.40 (2.3–3.05)	0 (0)	0 (0)	0 (0)	7, 8, 18, 22
Hen egg albumin	0.65 (0–1.48)	2.41 (2.0–2.40)	0 (0)	0 (0)	0.22 –	7, 23
Alpha-1-acid glycoprotein	7.72 (6.5–7.6)	5.82 (4.8–5.5)	0 (0)	0.64 (0.7–1.1)	0 –	24, 25
Bovine submaxillary mucin	1.00 (1.0–3.6)	0.54 (0–0.21)	0.11 (0)	0.30 (0.5–1.8)	0	7, 9, 26
Human saliva glycoproteins CTAB insoluble						
2800K MW fraction	12.6	0.9	0.2	6.6	0	
280K MW fraction	11.2	1.8	0.3	6.6	0	
30K MW fraction	2.8	1.1	0.5	1.5	0	
	(17.1)	(0.9)	(0.4)	(9.0)	(2.5)	19
Bovine skin proteodermatan sulphate	1.8	1.8	0.2	0.3	–	

* Literature values given in parentheses.

** Abbreviations: Gal = galactose; Man = mannose; Xyl = xylose; Fuc = fucose; Glc = glucose.

Human salivary glycoproteins were fractionated by precipitation with cetyltrimethylammonium bromide followed by size-exclusion HPLC on Merck Fractogel TSK[12] of the precipitated components. The neutral sugar composition of the high-molecular-weight salivary glycoprotein fractions (Fig. 1C, Table III) was quite similar to that of a fraction containing very large human salivary glycoproteins purified by methods differing from ours and analysed by methanolysis and GLC[19] instead of hydrolysis, dansylation and HPLC. The occurrence of significant amounts of mannose in most preparations of high-molecular-weight salivary glycoproteins confirms the presence of both N-linked and O-linked oligosaccharides in salivary glycoproteins[20,21].

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