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Liquid chromatographic method for the determination of the carbohydrate moiety of glycoproteins

Application to α_1-acid glycoprotein and tissue plasminogen activator

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ABSTRACT

A rapid procedure is described for the qualitative and quantitative analysis of the carbohydrate composition of glycoproteins by liquid chromatography with light-scattering detection. The analysis was carried out in three steps. First, the glycoprotein samples were purified by a two-step purification on a Sephadex G-25 column with a 90% yield. Second, the selectivity of the separation and the sensitivity of detection of monosaccharides, as methyl glycosides obtained by direct methanolysis of glycoproteins, were improved by modified simplex optimization of the methanolysis parameters (temperature, methanolic hydrochloric acid strength and reaction time) determined at 66°C, 1.2 *M* and 8.1 h for α_1-acid glycoprotein (α-AGP) and 73°C, 1.5 *M* and 12.5 h for tissue plasminogen activator (tPA). Finally, the method was applied to the determination of the carbohydrate moiety of the two N-glycosylated glycoproteins α-AGP and tPA.

INTRODUCTION

Many methods have been proposed for the determination of the carbohydrate moieties of glycoproteins after acid hydrolysis [1–3] or methanolysis [4,5]. Methanolysis of glycoconjugates produces methylglycosides in a one-step procedure. However, gas chromatographic methods require other cumbersome derivatization steps and direct liquid chromatographic techniques are limited by the poor detectability of the methylglycosides.

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We have described previously a rapid method for the determination of neutral and amino sugars present in polysaccharides and glycoproteins [6]. A light-scattering detector, which is more sensitive than a refractive index detector, was used and was suitable for the detection of methylglycosides having weak UV absorption bands. Using this procedure, quantitative analysis was possible as the calibration graphs obtained for various methylglycosides showed a linear response in double logarithmic coordinates.

The purpose of this work was to improve this method to allow the determination of the carbohydrate moieties of various glycoproteins using methanolysis. The first step was to develop a purification method for the glycoproteins to allow a suitable methanolysis procedure. The second step was to determine the optimum conditions of methanolysis as numerous conditions have been reported for the determination of carbohydrates in glycoproteins [4,7,8]. This scatter of values suggests that different glycoproteins would probably need differing optimum conditions of methanolysis. The choice of the internal standard and the reproducibility of the method were also investigated. Finally, this method was applied to the determination of the carbohydrate moieties of α_1-acid glycoprotein (α-AGP) and tissue plasminogen activator (tPA).

EXPERIMENTAL

Apparatus and chromatographic conditions

The chromatographic separation was carried out using a Shimadzu LC 6A pump equipped with a Cunow DDL 11 light-scattering detector and a Spectra-Physics Chromjet integrator.

The column effluent was nebulized by a stream of nitrogen. The particles formed were passed through a heated evaporation tube to evaporate the solvent. The particles then crossed a light beam and the scattered light was collected at an angle of 120° by a photomultiplier. The optimal nebulization pressure of 25 p.s.i. and the evaporative setting temperature of 30°C were determined previously [6].

The chromatographic column was Spherisorb ODS 2, 5 μm (250 × 4.6 mm I.D.) (SFCC). Methylglycosides were eluted with water–methanol (97:3) at 0.5 ml min^{-1}.

Reagents

Methanolic hydrochloric acid (3 *M*) was purchased from Supelco and methanolic hydrochloric acid (8.4 *M*) was provided by Dr. Doussin (Laboratoire de Biochimie, Hôpital Intercommunal de Creteil, France).

Human α-AGP, monosaccharides and standards of methylglycosides were obtained from Sigma. Plasminogen activator in solution was kindly donated by Dr. Schluter (Karl Thomae). All other chemicals were of analytical reagent grade.

Glycoprotein purification

In order to remove irrelevant products such as salts, buffers or contaminants from the sample, glycoprotein solutions were applied to a Sephadex G-25 PD10 column (Pharmacia) and eluted with 10% acetic acid. Gel filtration was performed twice. Eluted fractions (800 μl) were monitored by Lowry assay [9] and phenol–sulphuric acid assay [10].

Thin-layer chromatography (TLC) of the eluted fractions was performed on silica gel G-60 plates (Merck) with *n*-butanol–acetic acid–water (60:20:20, w/w/w) as eluent and ninhydrin solution as the spray reagent.

Methanolysis procedures

The glycoprotein-containing fractions were lyophilized for 18 h and the lyophilizates were used to prepare 1 mg ml^{-1} solutions. Aliquots of glycoprotein solution (250 μl) together with the internal standard (lyxose) were then lyophilized and dried over P_2O_5 under vacuum. A mixture of 500 μl of methanolic hydrochloric acid and 125 μl of methyl acetate was then added. To avoid decomposition of the reagent during storage, fresh methanolic hydrochloric acid stored at −20°C in sealed ampoules was used for each methanolysis. Dry distilled methanol was used to prepare the methanolic hydrochloric acid reagent at various strengths.

Samples were heated at various temperatures and durations according to the modified simplex procedure described below. Thereafter, the acid was removed under a stream of nitrogen at room temperature for 30 min. Samples were either freshly dissolved in distilled water or stored at −20°C without no significant destruction of the monosaccharide derivatives.

A modified simplex procedure [11] was used to optimize the methanolysis parameters. Three parameters were investigated: temperature, methanolic hydrochloric acid strength and reaction time. The boundaries of each parameter were defined according to previous published data on methanolysis [8,12]: temperature 50–120°C, methanol hydrochloric acid strength 0.2–4.2 *M* and reaction time 1–30 h. Each experiment was evaluated by a criterion C_R which was designed to assess the quality of the chromatogram:

$$C_R = \prod_{i=1}^{i=n} H_i$$

where H_i is the height of the main peak for each derivatized monosaccharide.

RESULTS AND DISCUSSION

Glycoprotein purification

The presence of buffer salts or other contaminants in the glycoprotein samples does not interfere with methanolysis [12]. Nevertheless, the additives present in the tPA solution disturbed the chromatographic separation of the first-eluted methyl glycosides released by methanolysis.

As shown in Fig. 1, contaminants could be removed from the glycoprotein solution by means of two consecutive gel filtrations on Sephadex G-25 with 10% acetic acid.

The elution of the glycoprotein was monitored both by the Lowry assay (for the peptidic fraction) and phenol–sulphuric acid reaction (for the glycan moiety); it was observed between the third and sixth eluted fractions.

Some interference with arginine, used in the buffer medium, was noted in the Lowry assay and allowed us to follow the elimination of arginine. The presence of

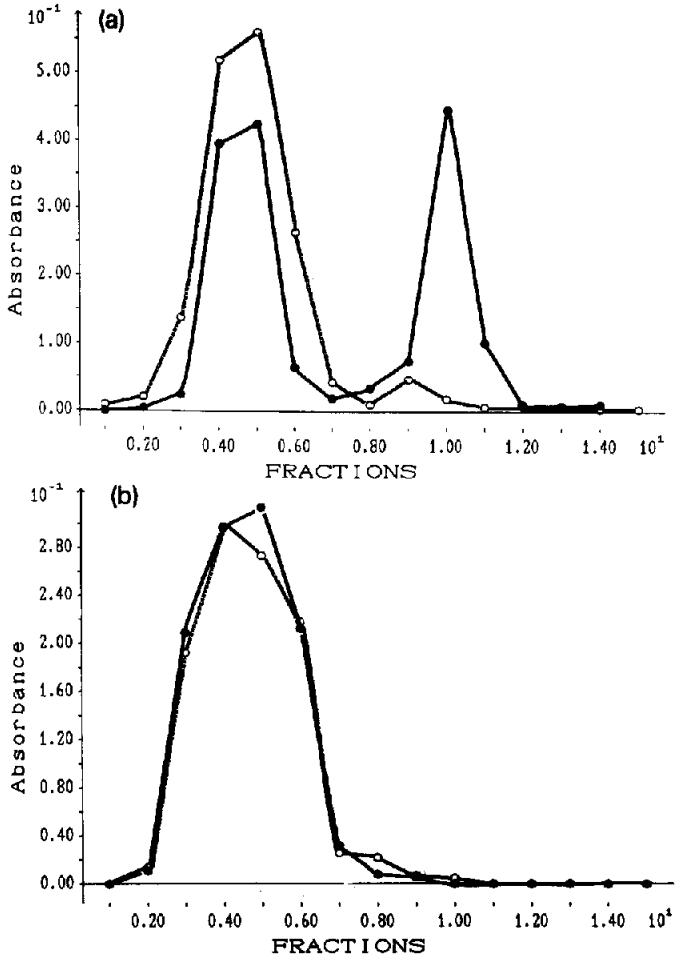

Fig. 1. Purification by gel filtration of tPA: (a) = step 1; (b) = step 2. Injected volume: 2 ml of a 1 mg ml^{-1} glycoprotein solution. Glycoprotein was monitored by (●) Lowry assay and by (○) phenol-sulphuric acid reagent.

residual arginine in the eluted fractions was checked by TLC and it was found to be present from fraction 9 to fraction 12.

The absorbance of the ninth fraction could be attributed to the presence of Tween 80 which interfered, to a minor extent, in the phenol–sulphuric acid assay.

The proposed two-step purification on Sephadex G-25 led to purified glycoprotein in 90% yield.

Simplex procedure

Methanolysis of a sugar produces a mixture of anomeric forms of methylglycosides and the anomer ratio is a function of the conditions used in the methanolysis step [13]. To obtain both good selectivity and sensitivity of the method, it was necessary to determine the methanolysis conditions which gave a main chromatographic peak per sugar.

To assess the quality of each chromatogram, we used the criterion C_R, which incorporates the product of the height of the main peak for each derivatized sugar. The product of H_i shows a better performance than the sum of H_i in which a small value for one peak can be compensated for by a great response of another peak. This product reflects an equal importance for each sugar as one bad response can lead to a dramatic decrease in the criterion.

The simplex procedure was stopped after five and seven steps for α-AGP and tPA, respectively. In the initial step of the experiments, the 50°C–0.2 *M*–1 h point was the first rejected point for both glycoproteins as these conditions were not sufficient to allow the methanolysis reaction. The extreme values for the three parameters studied, except the 4.1 *M* methanolic acid strength with α-AGP, were successively eliminated during the simplex evolution, leading to intermediate parameter values. This evolution causes the simplex to diminish in size (Fig. 2).

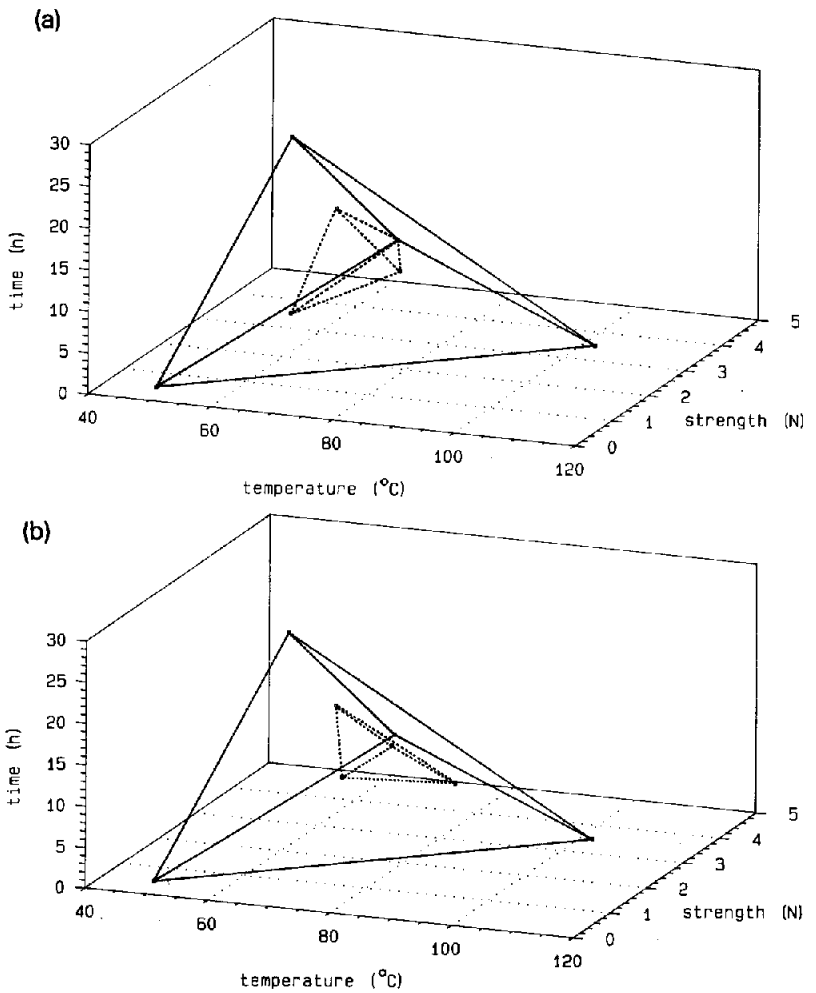

Fig. 2. Progress of the three-dimensional simplex towards the optimum for (a) α-AGP and (b) tPA.

Based on the results and the feasibility of the process, the optimum conditions established are reported in Table I. In order to compare the optimum conditions for α-GPA with those already published [8], a methanolysis of α-AGP was also carried out at 70°C with 0.6 *M* acid for 16 h. The criterion was improved by 50% with the new conditions.

Assuming that these two glycoproteins have similar N-glycan chains (high mannose, hybrid and complex types), the differences in the optimum point could be assigned to a dissimilarity in their spatial configuration rather than a different resistance of the glycosidic bonds to the methanolic hydrochloric acid. These results provide further evidence that optimum methanolysis conditions should be determined for each glycoprotein. The modified simplex procedure could be a simple approach for the optimization of these parameters in which variables such as temperature, reagent strength and reaction time, interact.

TABLE I

OPTIMUM CONDITIONS FOR THE METHANOLYSIS OF α-AGP AND tPA

Regression data in logarithmic coordinates for methylglycosides released from the glycoproteins by methanolyses under optimum conditions.

Methylglycoside	Methanolysis conditions					
	73°C, 1.5 *M*, 12.5 h			66°C, 1.2 *M*, 8.15 h		
	Slope	Intercept	Correlation coefficient	Slope	Intercept	Correlation coefficient
Galactose	1.264	4.548	0.997	1.448	3.783	0.999
Mannose	1.166	4.173	0.999	1.448	3.738	0.998
N-Acetylglucosamine	1.088	3.425	0.999	1.348	3.375	0.998
Fucose	1.050	3.506	0.996	1.422	2.712	0.998

Chromatographic application

A typical chromatogram of the separation of all methylglycosides including neutral and amino sugars is presented in Fig. 3. All the compounds are eluted within 16 min with good repeatability of the retention times [relative standard deviation (R.S.D.) ranging from 0.16 to 0.71%].

In order to choose an appropriate internal standard, we studied the repeatability of the chromatographic method with two different compounds, mesoinositol and lyxose. Two mixtures of methylglycosides containing either mesoinositol or lyxose as internal standards were analysed. The repeatibility of the method was evaluated by measuring the ratio of the peak height of each sugar to that of the internal standard with nine consecutive injections. The results obtained with both internal standards are reported in Table II. The average R.S.D. for all sugar derivatives was 3.9% with mesoinositol and 3.3% with lyxose. The R.S.D. values were scattered when mesoinositol was used as the internal standard. This could be attributed to its fast elution (retention time, t_R = 5.8 min), leading to a worse repeatability of peak heights for the last peaks. Moreover, mesoinositol has the disadvantage of being an underivatizable compound. In fact, the use of a methylglycoside for standardization provides a more

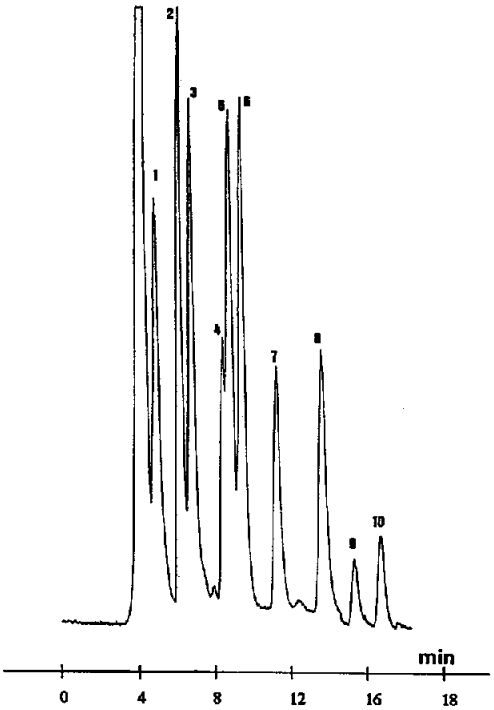

Fig. 3. Chromatographic separation of a mixture of methylglycosides. Detector parameters: atomiser inlet pressure, 25 p.s.i.; temperature, 30°C. Peaks: 1 = mesoinositol; 2 = methyl α-D-galactopyranoside; 3 = methyl α-D-glucopyranoside; 4 = methyl β-D-xylopyranoside; 5 = methyl α-D-mannopyranoside; 6 = methyl-D-lyxose; 7 = methyl α-D-N-acetylgalactosamine; 8 = methyl α-D-N-acetylglucosamine; 9 = methyl α-L-fucopyranoside; 10 = methyl α-L-rhamnopyranoside. Injections: 20 μl of 50 μg/ml of each solute. Attenuation: 256.

TABLE II

REPEATABILITY OF PEAK HEIGHT USING EITHER MESOINOSITOL OR LYXOSE AS INTERNAL STANDARD

Methylglycoside	R.S.D. (%)[a]	
	Lyxose as internal standard	Mesoinositol as internal standard
Galactose	3.7	4.7
Glucose	3.0	2.4
Mannose	3.5	1.3
N-Acetylglucosamine	3.6	3.3
Fucose	3.9	6.5
Rhamnose	2.4	5.2
Average	3.3	3.9

[a] $n = 9$.

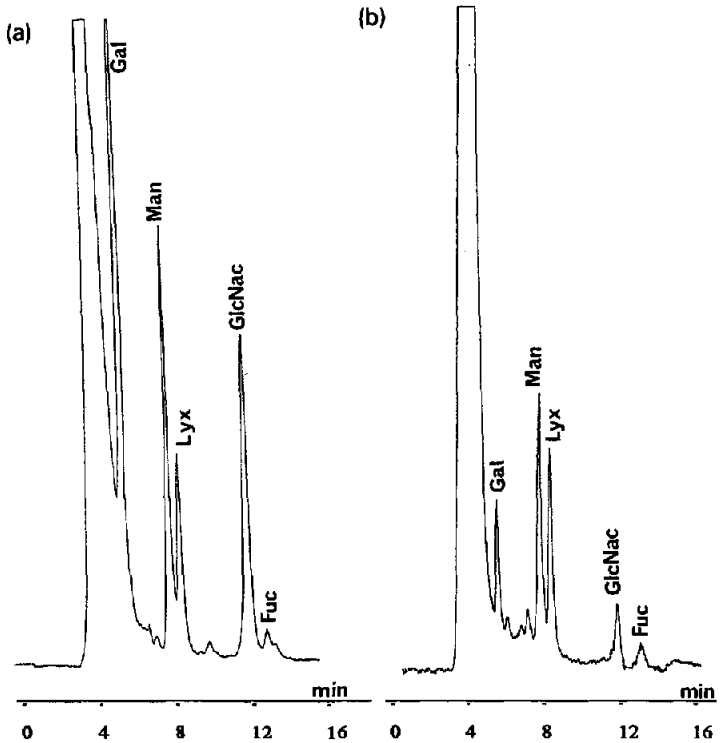

Fig. 4. Chromatograms of methylglycosides released from (a) α-AGP and (b) tPA by methanolysis using optimum methanolysis conditions. Detector parameters: atomiser inlet pressure, 25 p.s.i.; temperature, 30°C, injected volume: 20 μl, corresponding to 100 μg of the glycoprotein. Gal = galactose; Man = mannose; Lyx = lyxose; GlcNac = N-acetylglucosamine; Fuc = fucose. Attenuation: (a) 512, (b) 256.

rigorous test for the methanolysis procedure than the use of a free sugar. In contrast, lyxose seemed to be a more suitable internal standard in terms of repeatability. Methanolysis could be carefully monitored with respect to lyxose which was derivatized simultaneously with the glycoprotein sample. Lyxose gave rise to main peak which

TABLE III

MONOSACCHARIDIC COMPOSITION OF α-AGP AND tPA DETERMINED BY METHANOLYSIS FOLLOWED BY CHROMATOGRAPHIC ANALYSIS

Values expressed as mean ± S.D. in mole of sugar/mole of glycoprotein. tPA: MW = 66 000; α-AGP: MW = 44 000.

Component	Plasminogen activator[a]	α-AGP[b]
Galactose	3.9 ± 0.3	18.5 ± 1.4
Mannose	11.0 ± 0.5	15.9 ± 0.9
N-Acetylglucosamine	5.9 ± 0.7	26.1 ± 2.7
Fucose	2.1 ± 0.1	2.3 ± 0.1

[a] $n = 6$.
[b] $n = 3$.

was eluted intermediately (t_R = 8.25 min) just after methylmannose. A secondary anomer was also formed (t_R = 7.5 min) but it did not interfere with other sugars.

The method was applied to the determination of carbohydrate moiety of the two glycoproteins α-AGP and purified tPA using the conditions of methanolysis established above. Galactose, mannose, N-acetylglucosamine and fucose were readily identified in both glycoproteins (Fig. 4). In both glycoproteins, no significant amount of N-acetylgalactosamine was found, suggesting the absence of O-linked oligosaccharide. The results showed that tPA contained a high proportion of mannose, which points to the presence of mainly oligomannosidic or hybrid-type N-glycosidic chains. Moreover, the high content of N-acetylglucosamine in α-AGP indicates that N-linked oligosaccharides consist mainly of the N-acetyllactosamine type.

The reproducibility was investigated by carrying out the entire procedure (methanolysis followed by chromatographic analysis) nine times for the α-AGP and five times for the plasminogen activator. The results, expressed as the peak height of each derivative relative to that of methyllyxose, were satisfactory for all the monosaccharides present in the two glycoproteins, the R.S.D. ranging from 8.1 to 9.4% and from 4.6 to 11.2% for α-AGP and tPA, respectively.

Quantitative analysis was performed with a calibration using four concentrations (5–40 μg per 100 μl) of each monosaccharide. The peak heights were related to a constant lyxose peak height. As shown in Table I, the plots of peak height *versus* sample concentration in double logarithmic coordinates are linear. A four-point calibration graph is more accurate when dealing with unknown glycoproteins, but a two-point method would be sufficient when some data on monosaccharidic composition are already known.

The results obtained from the quantitative analysis are summarized in Table III and the quantification of each monosaccharide was found to be in a reasonable agreement with previously published data [2–4, 14–16].

This method, if applied more specifically to purified glycopeptides, could provide interesting information on the nature of the glycosidic chains attached to each glycosylation site of a glycoprotein.

CONCLUSIONS

It has been shown that the modified simplex procedure could be useful for optimizing rapidly the parameters involved in methanolysis such as temperature, reaction time and methanolic hydrochloric acid strength, as these factors are numerous and interdependent.

The entire technique, which involves the cleavage of glycosidic linkages by methanolysis of a purified glycoprotein and the determination of the released methylglycosides by liquid chromatography, is a rapid, sensitive and reproducible method for the determination of monosaccharidic maps from glycoproteins. The carbohydrate compositions of α-AGP and tPA obtained by this method closely match the compositions previously reported with other analytical techniques.

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