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SENSITIVE METHOD FOR CARBOHYDRATE COMPOSITION ANALYSIS OF GLYCOPROTEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new high-performance liquid chromatographic system has been developed for separating completely the neutral alditols and aminoalditols derived from sugars in complex carbohydrates. In combination with reduction by NaB^3H_4, this system affords a very sensitive and rapid method of monosaccharide composition analysis of glycoproteins and glycolipids.

INTRODUCTION

Sugar moieties of complex carbohydrates have been shown to play important rôle in many physiological processes, such as recognition signals[1–3]. They also serve as immunodeterminants of several antigens, including tumour-related antigens[4] as well as differentiation antigens[5], and contribute to the conformational stabilization of glycoproteins against proteolytic degradation and denaturation. In order to elucidate such rôles, the establishment of a series of sensitive methods for structural studies is essential.

We had developed a sensitive method for the determination of the monosaccharide composition of sugar chains[6]. In this method, the amount of each monosaccharide in an acid hydrolysate is determined after conversion into tritium-labelled sugar alcohols by reduction with NaB^3H_4 and separation by paper electrophoresis using a borate buffer. Although inexpensive, the method is inconvenient for the analysis of a large number of samples because it is time-consuming.

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In order to overcome this problem, we have developed a high-performance liquid chromatographic (HPLC) method for fractionating all the sugar alcohols derived from monosaccharides found in glycoproteins and glycolipids. This novel method was successfully applied to the analysis of the sugar composition of erythropoietin samples produced by recombinant Chinese hamster ovary (CHO) cells as well as natural glycoproteins.

EXPERIMENTAL

Materials

If not specified, reagents were obtained from Nakarai (Kyoto, Japan). D-Allose, 2-deoxy-D-galactose and D(+)-talose were obtained from Fluka (Buchs, Switzerland), L(−)-idose from Sigma (St. Louis, MO, U.S.A.), D-quinobose from Koch-Light Lab. (Haverhill, U.K.) and bovine fetuin (Type III) from Sigma. Human erythropoietin, produced in recombinant CHO cells (rec h EPO), was kindly donated by KIRIN-Amgen (Thousand Oaks, CA, U.S.A.). Ion-exchange resins, AG 3-X4A (100–200 mesh) and AG 50W-X12 (100–200 mesh), were from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Sodium borotritide, NaB^3H_4 (340 mCi/mmol), was obtained from New England Nuclear (Boston, MA, U.S.A.).

Monosaccharide alcohols were prepared by reduction of the respective monosaccharides by sodium borohydride, as described previously[6].

Acid hydrolysis

Approximately 1 nmol of a glycoprotein was hydrolyzed with 0.5 ml of either 2 or 4 *M* hydrochloric acid in a sealed tube at 100°C in an oil-bath for 0–30 h. The hydrolysates were then evaporated to dryness, the residues freed from acid by evaporation three times with water and then by keeping in a desiccator over sodium hydroxide *in vacuo*, overnight.

Labelling of sugars by reduction with NaB^3H_4 and re-N-acetylation

The procedure described in our previous paper[6] was slightly modified. The acid hydrolysate was incubated at 30°C for 4 h in 100 μl of 0.05 *M* sodium hydroxide containing a 5-molar excess amount of NaB^3H_4. At the end of the incubation, 5 mg of sodium borohydride were added to the solution. After incubation for another 30 min, the reaction was stopped by addition of 200 μl of 1 *M* acetic acid. An 800-μl volume of saturated sodium bicarbonate solution was added. The labelled sugars were then re-N-acetylated by adding 10 μl of acetic anhydride and incubating at room temperature for 10 min. This N-acetylation reaction was repeated four more times by adding 200 μl of saturated sodium bicarbonate solution and 10 μl of acetic anhydride to the solution each time. An excess amount of acetic anhydride was added to release carbon dioxide gas and to complete the re-N-acetylation. The solution was passed through an ion-exchange column containing 2.5 ml of AG 50W-X12 (H^+, 100–200 mesh) in the upper layer and 2.5 ml of AG 3-X4A (OH^-, 100–200 mesh) in the lower layer. The column was washed with 20 ml of deionized distilled water. The eluate and washings were pooled and evaporated to dryness. To the residue, 1 ml of methanol was added and then evaporated to dryness. This procedure was repeated

four more times completely to remove boric acid. Finally, the labelled sugars were dissolved in 50 μl of 20% aqueous ethanol, containing 1% each of non-labelled N-acetylgalactosaminitol ($GalNAc_{OH}$), N-acetylglucosaminitol ($GlcNAc_{OH}$), mannitol (Man_{OH}), fucitol (Fuc_{OH}), galactitol (Gal_{OH}), glucitol or sorbitol (Glc_{OH}) and 2-deoxyribitol ($2DR_{OH}$).

Standard conditions for separation of alditols and aminoalditols

A Shodex SUGAR SP-1010 column with a SP-1010P pre-column and A Shodex Degas, on-line degasser (Showa Denko, Tokyo, Japan) were installed in a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). The column was kept at 80°C by the column heater (Model 1122; Waters Assoc., Milford, MA, U.S.A.). An aliquot of the sample solution (10 μl) described in the previous section was injected into the column and eluted isocratically with 20% (v/v) ethanol in distilled water at 0.5 ml/min. A differential refractometer, Shodex RI SE-51 (Showa Denko), and a Radio Analyzer RLC-551 (Aloka, Tokyo, Japan) were used for monitoring the non-radioactive and radioactive sugar alcohol standards, respectively, eluted from the column.

RESULTS AND DISCUSSION

Selection of a column for HPLC

Although several types of columns for the analysis of reducing sugars are now available, their usefulness for the separation of sugar alcohols has not been studied in detail. Therefore, we studied the elution profiles of the four neutral alditols (Man_{OH}, Fuc_{OH}, Gal_{OH} and Glc_{OH}) and the two aminoalditols ($GlcNAc_{OH}$ and $GalNAc_{OH}$) that commonly occur in glycoproteins and glycolipids. Our preliminary studies revealed that reversed-phase and gel-permeation columns do not separate the six sugars effectively. A column of Shodex SUGAR SP-1010, operating in the gel-permeation plus ligand-exchange mode with Pb^{2+} as cation, gave a relatively good separation of individual neutral alditols and aminoalditols. Therefore, we selected this column and searched for conditions with which to obtain complete separation of the six sugars.

Separation of sugar alcohols by HPLC

The six standard sugars were separated on the Shodex SUGAR SP-1010 column by isocratic elution with ethanol or acetonitrile at different concentrations in water at 80°C. Elution with water gave a fairly good separation, except for Fuc_{OH} and Gal_{OH}, which were not completely separated. Addition of increasing amounts of acetonitrile to the eluent somewhat improved the resolution, but the separation of $GalNAc_{OH}$, $GlcNAc_{OH}$ and Man_{OH} was not complete. Much better separations of the six sugars were obtained by addition of increasing amounts of ethanol to the eluent. At 20% ethanol, a baseline separation of the sugars was obtained. Consequently, we adopted 20% aqueous ethanol as the standard eluent. It must be stressed here that ethanol is preferable to acetonitrile because the latter is toxic.

Sugar alcohols other than the six described above were also subjected to HPLC analysis under the standard condition in order to select an appropriate internal standard for the labelling efficiency. The results are summarized in Table I in terms of

TABLE I

RETENTION TIMES OF SUGAR ALCOHOLS ON A SHODEX SUGAR SP-1010 COLUMN

Group	*Sugar alcohol*	*Retention time (min)*	*Group*	*Sugar alcohol*	*Retention time (min)*
Hexitols	Allositol	37.40	6-Deoxyhexitols	Rhamnitol	46.00
	Mannitol	46.38		Fucitol	53.94
	Talitol	50.22		Quinobitol	63.90
	Galactitol	61.56	N-Acetylamino-hexitols	N-Acetylmannos-aminitol	33.66
	Sorbitol	67.62		N-Acetylgalactos-aminitol	33.90
	Iditol	83.94		N-Acetylglucos-aminitol	39.00
Pentitols	Ribitol	35.10	Others	2-Deoxyribitol	27.00
	Arabinitol	44.40		Glycerol	29.64
	Xylitol	55.40			

retention times. Among the sugar alcohols tested, 2-deoxyribitol had a quite different retention time from those of $GalNAc_{OH}$, $GlcNAc_{OH}$, Man_{OH}, Fuc_{OH}, Gal_{OH} and Glc_{OH}. Therefore, we decided to use 2-deoxyribose as the internal standard, added to the acid hydrolysate before reduction by NaB^3H_4, in order to obtain the labelling efficiency for calculating the actual content of each sugar component.

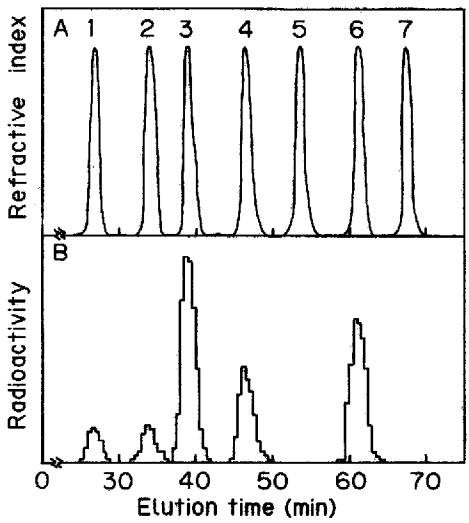

Fig. 1. The elution pattern of alditols and aminoalditols from fetuin obtained with the HPLC system. Alditols, obtained from fetuin by hydrolysis with 4 *M* hydrochloric acid at 100°C for 3 h, were mixed with 10 μl of sugar alcohol standard solution, and injected into the HPLC system. Chromatography was performed under the standard conditions (see text). Panels A and B show the elution patterns of sugar alcohol standards and labelled alditols, respectively. Peaks: 1 = $2DR_{OH}$; 2 = $GalNAc_{OH}$; 3 = $GlcNAc_{OH}$; 4 = Man_{OH}; 5 = Fuc_{OH}; 6 = Gal_{OH}; 7 = Glc_{OH}.

TABLE II

COMPARISON OF THE MONOSACCHARIDE COMPOSITIONS DETERMINED BY PAPER ELECTROPHORESIS (PEP) AND HPLC

Fetuin (50 μg) was hydrolyzed with 4 *M* hydrochloric acid for 3 h. The released saccharides were labeled with NaB^3H_4, as described in Experimental, and the labelled sugars were analyzed by paper electrophoresis[6] and the present HPLC system. The values are expressed in mol/mol of fetuin.

Method	*Amino sugars*		*Neutral sugars*	
	GalNAc	*GlcNAc*	*Man*	*Gal*
PEP	2.36	16.5	8.33	11.8
HPLC	2.50	16.4	8.30	12.6

The recovery of all sugar alcohols was above 98%. Reinjection of each sample gave the same chromatogram with the same recovery indicating that adsorption, degradation and isomerization did not occur during chromatography.

Verification of HPLC system for sugar composition analysis

The usefulness of the HPLC system for actual sugar composition analysis was tested by using fetuin, which has already been well studied[7]. A labelled sample, obtained by acid hydrolysis of fetuin with 4 *M* hydrochloric acid at 100°C for 3 h, was subjected to HPLC analysis. As shown in Fig. 1B, the labelled sugar alcohols were monitored and identified by comparing their retention times with those of the standard sugar alcohols (Fig. 1A), since co-injection of the radiolabelled sugar alcohols and the non-labelled standard sugar alcohols did not affect the retention time of either of them (data not shown). The radioactivity incorporated into each component was measured by liquid scintillation counting. Based on the radioactivity incorporated into the internal standard, 2-deoxyribose, the contents of the individual monosaccharides in 1 mol of fetuin were calculated (Table II). Data on the same sample, obtained by the earlier method based on borate paper electrophoresis[6], are also shown. The values determined by the two methods are quite comparable, indicating

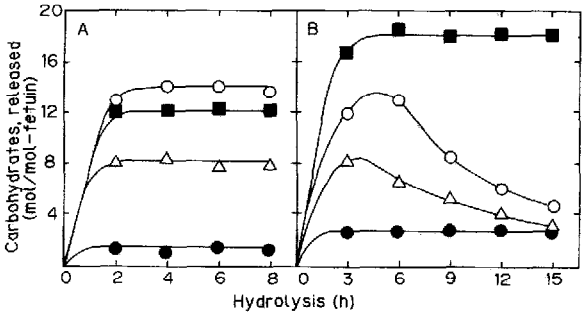

Fig. 2. Liberation of monosaccharides from fetuin by acid hydrolysis. Fetuin (50 μg) was hydrolyzed for different incubation periods with 500 μl of 2 (A) or 4 *M* hydrochloric acid (B) in a sealed tube at 100°C in an oil-bath. The monosaccharides released were analyzed in the HPLC system under the standard conditions (see text). ○, Gal; △, Man; ■, GlcNAc; ●, GalNAc.

that the HPLC system now developed can be used for the sugar composition analysis of glycoproteins as effectively as the radioelectrophoretic method, but is much faster than the latter.

The values were not exactly the same as those previously reported by Spiro and Bhoyroo[7]. This discrepancy could be the result of different conditions of acid hydrolysis. Therefore, we carried out a time-course study of acid hydrolysis of the glycoprotein. The release of monosaccharides from fetuin by acid hydrolysis with 2 and 4 *M* hydrochloric acid is shown in Fig. 2A and B, respectively. Bovine fetuin has three N-linked sugar chains, which are mainly the triantennary complex-type sugar chains and a small amount of biantennary ones, and three O-linked chains[7]. Galactose (Gal) and mannose (Man) liberated from fetuin reached their maximum values of 13.1 and 8.30 mol/mol of fetuin, respectively, by hydrolysis with 2 *M* hydrochloric acid for 2 h. Both values were slightly decreased after hydrolysis for 30 h (data not shown). Only part of the N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) was released under these conditions. In contrast, GalNAc and GlcNAc reached their maximum values, 2.90 and 18.3 mol/mol of fetuin, respectively, by hydrolysis with 4 *M* hydrochloric acid for 6 h. These values remained constant until 15 h of hydrolysis, but decreased to 50% after heating for 30 h (data not shown). Gal and Man were quickly destroyed under these conditions. The carbohydrate composition of fetuin, determined by the HPLC system, is in accordance with that reported previously (GalNAc, 3.1; GlcNAc, 15.7; Man, 8.1; Gal, 12.4 mol/mol of fetuin)[7].

Determination of the carbohydrate composition of erythropoietin produced by recombinant mamalian cells

Although the carbohydrate moiety of glycoprotein hormones and bioactive substances is known greatly to affect their biological activities, analysis of the carbohydrate moieties of the glycoproteins produced by recombinant methods has scarcely been performed. Since the method described in this paper is expected to be useful for such a study, we tried to analyze the sugar composition of erythropoetin obtained by the recombinant method (rec h EPO) reported by Lin *et al.*[8]. As shown in Fig. 3A and B, rec h EPO contains 1.40, 16.0, 7.90, 2.40 and 11.9 mol of GalNAc,

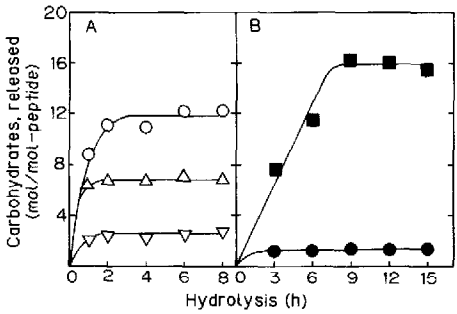

Fig. 3. Carbohydrate composition analysis of rec h EPO with the HPLC system. rec h EPO (30 μg) was hydrolyzed for different incubation periods with 2 (A) or 4 *M* hydrochloric acid (B), and the monosaccharides released were analyzed in the HPLC system under the standard conditions (see text). ○, Gal; △, Man; ▽, Fuc; ■, GlcNAc; ●, GalNAc.

GlcNAc, Man, fucose (Fuc) and Gal, respectively, per mole of rec h EPO. On the basis of the composition thus obtained and the rule that complex-type sugar chains commonly contain three mannosyl residues in each chain, it was suggested that the rec h EPO has three fucosylated complex-type sugar chains and one O-linked sugar chain. This assumption is in accordance with the finding by Egrie *et al.*[9] that rec h EPO gave additional three bands in sodium dodecyl sulphate polyacrylamide gel electrophoresis after partial N-glycanase digestion and that the band of rec h EPO moved slightly after O-glycanase digestion.

The HPLC system described in this paper should be suitable for the analysis of the carbohydrate moieties of other glycoprotein samples produced by recombinant mammalian cells since it requires less than 1 nmol of glycoproteins.

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