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Single-step isolation method for six glycoforms of human α_1-acid glycoprotein by hydroxylapatite chromatography and study of their binding capacities for disopyramide

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Abstract

A single-step isolation method for the glycoforms of human serum α_1-acid glycoprotein (AAG) using a hydroxylapatite column under a gradient elution program was developed. The concentrations of *N*-acetylneuraminic acid and monosaccharides (fucose, *N*-acetylglucosamine, galactose and mannose) of six AAG glycoforms were determined by the pulsed-amperometric detection method. For each AAG glycoform, significant sex-related differences in carbohydrate content have been observed only for AAG glycoforms two and six, and not for each AAG glycoform. The relationship between the extent of the branch in the glycan chain and the binding capacity to disopyramide were examined. Female AAG contained highly sialylated AAG glycoforms compared to male glycoforms. Conversely, male AAG was rich in the lower sialylated AAG glycoform. Furthermore, it was found that the drug binding capacity decreases with increasing branching of the glycan chain. This suggests that the binding sites of AAG are hindered by a relatively large carbohydrate moiety, such as tetraantennary structures.

Keywords: α_1-Acid glycoprotein

1. Introduction

α_1-Acid glycoprotein (AAG) is a characteristic and dominant fraction of human sialoglycoproteins with a molecular mass of 44 kDa, an unusually high carbohydrate content (41%) and a large number of sialyl residues. Although its exact biological function is still unknown, AAG is an acute-phase reactant that increases under several physiological and pathological conditions [1]. AAG has also been identified in plasma as a transporter protein for many basic drugs [1]. Since the existence of variants of AAG was demonstrated in 1962 [2], a high degree of heterogeneity of human AAG has been found. Several attempts have been made to elucidate the structure–function relationships, such as drug binding differences, between the variants [22]. Some of these studies used the slow (S-AAG) and/or fast (F-AAG)

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migrating variants after isoelectric focusing of desialylated AAG. More recently, Hervé et al. [8] isolated the three main variants (F1, S and A) (genetic variants) from native (sialylated) AAG by their newly developed affinity chromatography method, and observed differences in the binding of drugs to these variants [7]. There are very few reports, however, on the correlation between the structure of AAG glycoforms and the drug binding capacity. In the present study, we have developed a rapid single-step high-performance liquid chromatography (HPLC) system that is able to separate the six AAG glycoforms in sufficient amounts from AAG samples that were isolated previously from the plasma of healthy individuals, in order to study their carbohydrate moiety and their binding capacity for disopyramide. For the six AAG glycoforms obtained, sex-related differences in the carbohydrate content and the relationship between the extent of branching in the glycan chain and the drug (disopyramide) binding capacity were examined.

2. Experimental

2.1. Materials

The serum used in this study was obtained from ten healthy subjects (five men and five women, aged 22–26 years). Serum samples were frozen at −80°C until use. Human AAG (Lot 57F-9319), fucose and *N*-acetylglucosamine (GlcNAc) were purchased from Sigma (St. Louis, MO, USA). *N*-Acetylneuraminic acid (NeuAc), galactose and mannose were obtained from Wako Pure Chemicals (Osaka, Japan). Disopyramide was kindly donated by Morishita Roussel (Osaka, Japan). All other reagents were of the highest grade available and were used without further purification.

2.2. Purification of AAG and isolation of AAG glycoforms

The purification of AAG was carried out using the chromatographic methods previously described [9]. Each serum sample (10–15 ml) was used as the starting sample for the purification procedure. Each purified AAG (ca. 8–10 mg) was dissolved in 0.5 ml of distilled water, and the whole volume was applied to the HPLC system (Model L-6200, Hitachi, Tokyo, Japan), equipped with a multi-wavelength UV detector (Hitachi, L-4000) set at 280 nm. The separation of AAG glycoforms was achieved on a hydroxylapatite column (F-2025, 250×21.4 mm I.D.; Koken, Tokyo, Japan) with a gradient program (Fig. 1) at a flow-rate of 9.5 ml/min and at room temperature. Then, 9.5 ml fractions were collected and the eluates were desalted on an Ampure SA column (55×15 mm I.D.; Amerham Japan, Tokyo, Japan), equilibrated with distilled water and lyophilized. The concentration of each AAG glycoform was determined by the HPLC method described previously [10].

2.3. Determination of NeuAc and monosaccharides in each AAG glycoform

Each AAG glycoform (0.3 mg) was dissolved in 0.3 ml of distilled water, and 0.1 ml was used for the determination of NeuAc and the concentration of monosaccharides, respectively. The procedure for the release of NeuAc, acid hydrolysis of carbohydrates, and the determination of NeuAc or monosaccharides were carried out as described previously [9]. Detection of NeuAc and monosaccharides was performed by high-pH anion-exchange chromatography with pulsed-amperometric detection (HPAEC–PAD), which was developed in this laboratory [9].

2.4. Drug binding capacity of AAG glycoforms

In order to determine the concentrations of AAG glycoforms and disopyramide in the serum, mixtures of each AAG glycoform (1 mg/ml) and disopyramide (5.0 μg/ml) were prepared in 0.01 *M* citrate phosphate buffer (pH 7.4). The drug binding capacity was determined by the ultrafiltration method using a Millipore ultrafiltration membrane (an Ultrafree C3 LGC, M_r cut-off of 10 000; Millipore, Bedford, MA, USA). Binding studies were carried out at 37°C, and the disopyramide concentration in the filtrate was determined by the HPLC method as described previously [11].

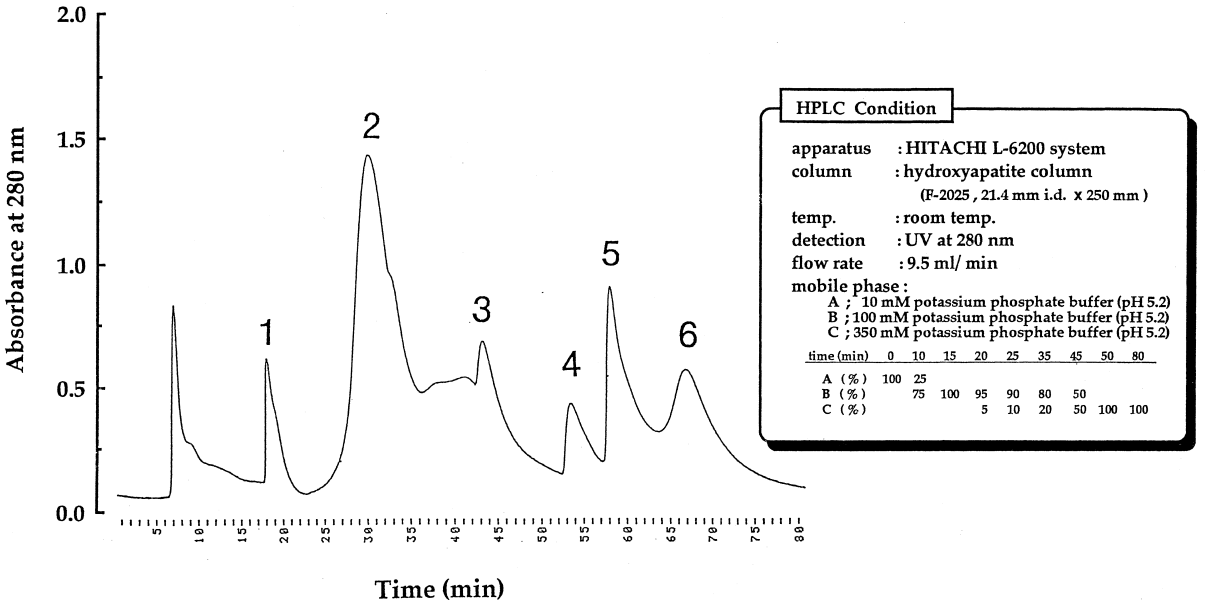

Fig. 1. Typical chromatogram of the glycoforms of α_1-acid glycoprotein from the serum of healthy subjects by HPLC. Inset is the gradient program for the fractionation of glycoforms of α_1-acid glycoprotein. (Sampling time of each fraction: fraction 1, 17–22 min; 2, 27–36 min; 3, 43–50 min; 4, 53–57 min; 5, 58–62 min and 6, 65–72 min, respectively).

2.5. Statistical analysis

The differences among the groups were examined by Student's *t*-test to determine the level of significance.

3. Results and discussion

3.1. Isolation of AAG glycoforms and their contents of carbohydrate

A typical chromatogram of AAG glycoforms from purified AAG is shown in Fig. 1. At least six glycoforms could be obtained by the gradient elution program, and the separation patterns of each of the ten subjects were almost the same. As shown in Fig. 2, glycoforms two and six are relatively abundant and, surprisingly, the contents of these two glycoforms were significantly different between men and women. In the standard AAG, on the other hand, the peaks of glycoforms five and six were much lower than those shown in Fig. 1.

Fig. 3 shows the contents of NeuAc and four monosaccharides (fucose, GlcNAc, galactose and mannose) in each AAG fraction. Glycoforms rich in carbohydrates were eluted when the ionic strength was increased (Chart 1, Fig. 1), and NeuAc was relatively abundant in these highly adsorbed glycoforms. The usefulness of the hydroxylapatite column in protein chromatography has been demonstrated

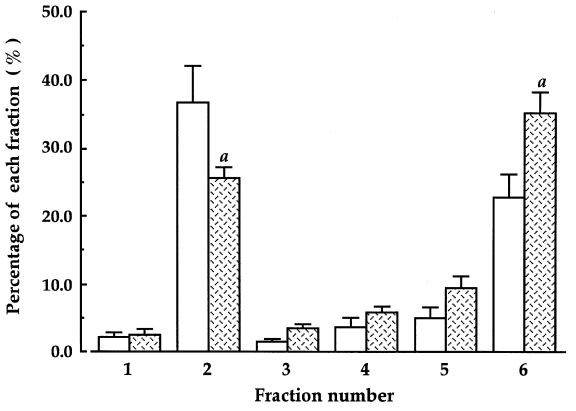

Fig. 2. Percentage of each variant of α_1-acid glycoprotein from the serum of healthy male and female subjects. (White bars) male; (Grey bars) female. [a]Significantly different ($p<0.05$) from that found in males.

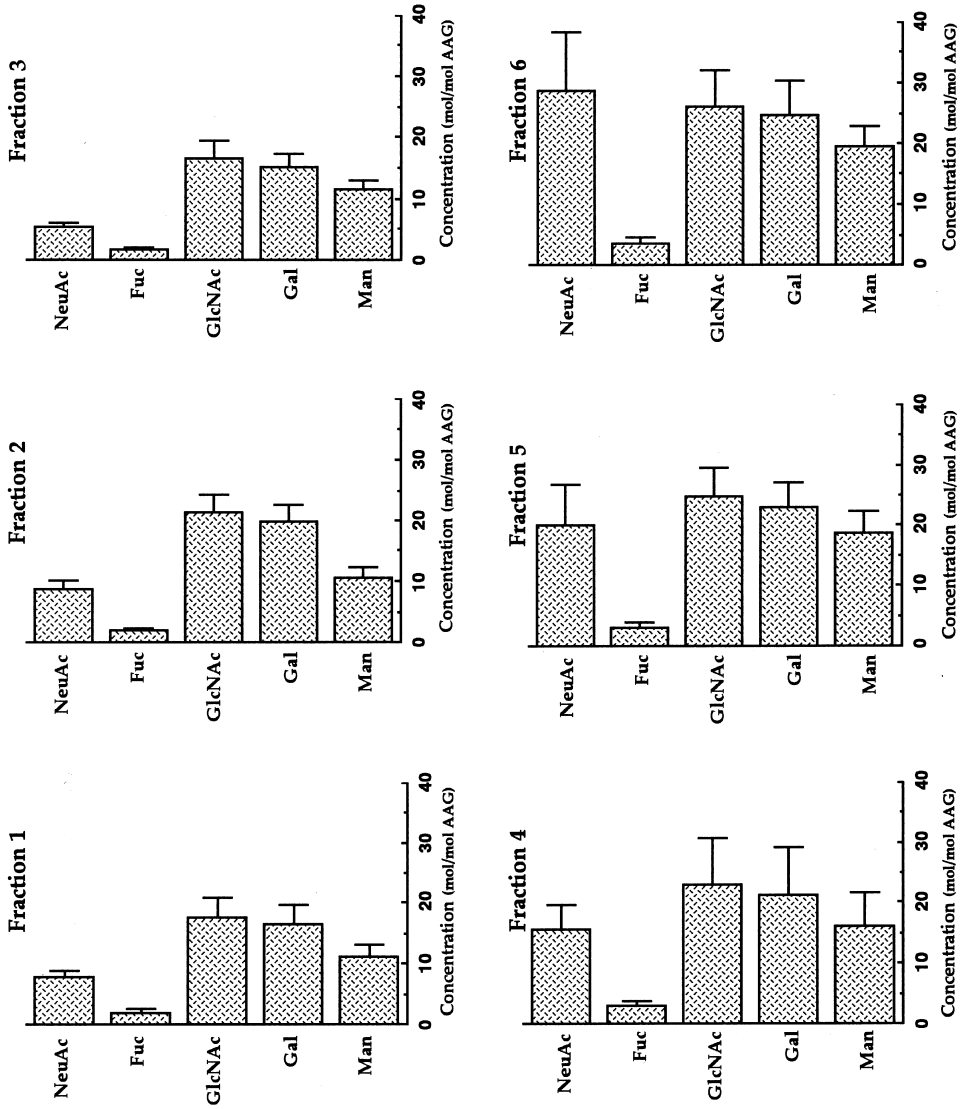

Fig. 3. Comparison of the contents of NeuAc and monosaccharides in each glycoform of α_1-acid glycoprotein from the serum of healthy subjects.

[12–15]. A good separation of AAG glycoforms is thought to be due mainly to the anion-exchange properties of this column [15].

It is interesting that female AAG contained the highly sialylated AAG glycoforms compared to male, while male AAG was rich in lower sialylated AAG glycoforms. Furthermore, the contents of highly sialylated AAG glycoforms of the standard AAG were much lower than those obtained from the Japanese (data not shown). It has been shown that tetra- and triantennary glycans obtained from normal AAG are more sialylated than biantennary glycans [16]. In contrast, in the present results, highly branched glycan forms, fractions 1 and 2, were less

sialylated. The reason for this discrepancy should be examined further.

3.2. Degree of branching of the glycan chain

In order to estimate the effect of glycans of AAG glycoforms on the drug binding capacity, the extent of branching of each AAG glycoform was examined. It is well known that the microheterogeneity of AAG is due to the occurrence of di-, tri- and tetraantennary glycans of the *N*-acetyllactosamine type at the five glycosylation sites [17]. Moreover, the mannose content is constant among the antennary glycans, and the number of branches increases with the addition of GlcNAc to mannose residues. A highly branched glycan chain of AAG is constructed by the linkage of galactose to GlcNAc, which results in the formation of an antennary structure (*N*-acetyllactosamine). Therefore, in the case of AAG, the determination of the concentration ratio of GlcNAc to mannose (GlcNAc/Man) is important for estimating if the carbohydrate moiety of glycoforms has a highly or less branched glycan chain. As shown in Fig. 4, it was found that the carbohydrate moieties of fractions 1 and 2, especially fraction 2, have a highly branched glycan chain compared to those of other fractions.

3.3. Binding activity of the glycoforms to disopyramide

The binding capacities of each AAG glycoform to disopyramide were compared (Fig. 5). Fractions 1 and 2 showed a significantly lower binding capacity than those of other fractions. On the other hand, the binding capacities of fractions 3–6 were almost the same, although the NeuAc contents differed significantly, indicating that the NeuAc residue is not involved in drug binding. We also compared the binding capacity of native AAG with that of asialo AAG, which was obtained by neuraminidase treatment [9]. The binding capacities of both native and asialo AAG to disopyramide were almost the same (data not shown). These observations agree well with previously reported results [18–20]. As shown in the relationship between the extent of branching and the binding capacity (Figs. 4 and 5), it was obvious that AAG glycoforms with a highly branched glycan chain have a lower binding capacity. This suggests that the binding sites of AAG are hindered by relatively large carbohydrate moieties, such as a tetraantennary structure. We previously reported that the affinity [the number of binding sites (*N*)] of

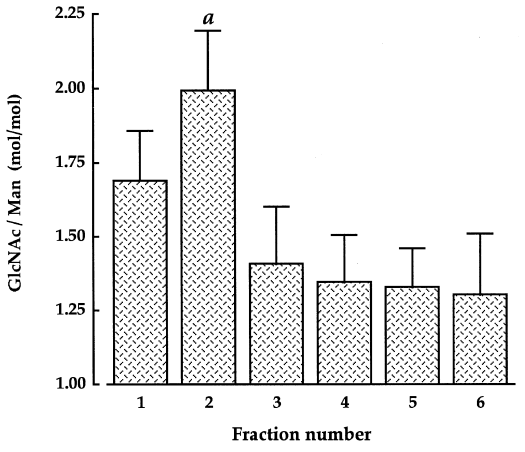

Fig. 4. Comparison of the molecular ratio of *N*-acetylglucosamine to mannose (GlcNAc/Man) in each glycoform of α_1-acid glycoprotein. [a]Significantly different ($p<0.05$) from fractions 3, 4, 5 and 6. Each column represents the mean±S.E.M.

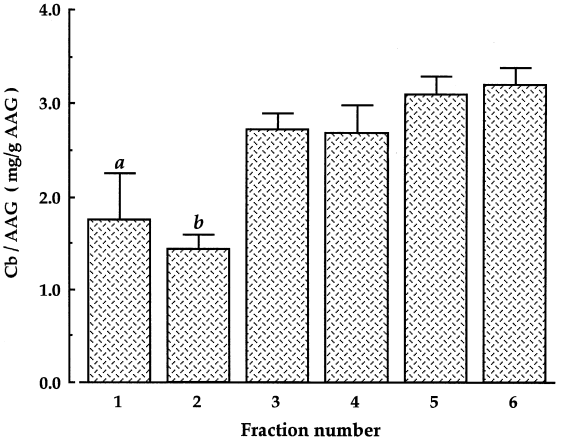

Fig. 5. Comparison of the amount of disopyramide bound to each glycoform of purified α_1-acid glycoprotein (Cb/AAG) from the serum of healthy subjects. [a]Significantly different ($p<0.05$) from fractions 3, 4, 5 and 6. [b]Significantly different ($p<0.01$) from fractions 3, 4, 5 and 6. Each column represents the mean±S.E.M.

purified AAG isolated from patients with renal insufficiency to disopyramide is significantly lower than that found in healthy subjects [21]. Furthermore, we observed that the contents of GlcNAc in purified AAG from patients with renal insufficiency [9] or myocardial infarction (unpublished data) were significantly higher than those in healthy subjects. These previous and present results suggest that AAG glycoforms with a highly branched glycan chain may be more abundant in a disease state.

Several investigators have studied the differences in the drug binding capacity among AAG variants [3–7]. Hervé et al. [7] isolated large amounts of the A variant in a pure form and a mixture of the F1 and S variants from native (sialylated) AAG, and investigated their drug binding properties. More recently, Hervé et al. [22] reported that disopyramide binds to only one of the AAG proteins/variants (A variants). In the present study, we obtained six AAG glycoforms by using an hydroxylapatite column under mild elution conditions. Analysis of the carbohydrates in each AAG glycoform revealed that the drug binding capacity decreases with an increase in the extent of branching of the glycan chains. We think that the six AAG glycoforms obtained using the proposed method are probably a mixture of different proteins/variants (the A and F1 and/or S variants). A comparison of the AAG glycoforms obtained in the present study with the glycoforms corresponding to the F1, S and A variants is necessary to gain further insight into the structure–binding relationship of the AAG glycoforms.

This simple and reproducible method for the isolation of large amounts of AAG glycoforms should be useful for estimating the correlation between the AAG glycoforms and drug binding capacity.

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