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Purification method for α-1-acid glycoprotein with subsequent high-performance liquid chromatographic determination of monosaccharides in plasma of healthy subjects and patients with renal insufficiency

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Abstract

A simple purification method for human plasma α-1-acid glycoprotein (AAG) using an ion-exchange and hydroxyapatite column was developed. The recovery of the method was found to be high. We also improved a determination method for N-acetylneuraminic acid and monosaccharides in the carbohydrate moiety of AAG by using an ion-exchange column and pulse-amperometric detection. By this method, a composition analysis of the carbohydrate moiety of AAG (N-acetylneuraminic acid, fucose, N-acetyl glucosamine, galactose and mannose) was possible with 1.0 ml of plasma. We compared these carbohydrate concentrations in the AAG of patients with renal insufficiency with those of healthy subjects. In the AAG of the patients, the concentrations of N-acetylglucosamine, galactose and mannose were significantly higher than those in the AAG of the healthy subjects.

1. Introduction

It is well known that the acute-phase reactant protein, α-1-acid glycoprotein (AAG), increases following cancer [1–3], renal insufficiency [4], myocardial infarction [5,6], and congestive heart failure [7]. The mechanism of its increase is not well understood. Several data obtained by electrophoresis have suggested that there is a change in the carbohydrate moiety of this protein in the disease-state [8,9]. Qualitative alterations in the oligosaccharide structure of AAG have been observed in some physiological and pathological states such as rheumatoid arthritis [10,11], liver disease [12] and acute inflammation [13]. Compositional analysis of the carbohydrate moiety of the glycoconjugates is of fundamental importance in structural studies of these compounds.

The key to studying the structural heterogeneity of AAG is to isolate the glycoprotein from

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plasma without inducing any structural degradations. Several purification methods for AAG have been reported. Earlier purification methods have used a combination of chromatographic procedures on DEAE- and/or CM-cellulose [14–16]. Gel filtration chromatography on Sephadex G-100 has been used for the final steps. These methods, however, are time-consuming and require a large volume of plasma or serum because of the low quantities recovered. Furthermore, there is a definite possibility that these methods will damage the oligosaccharide and polypeptide components. Recent successful procedures which avoid the desialylation and denaturation of AAG have utilized various affinity adsorbents [12,13,17] and dye–ligand chromatography [18]. These methods necessitate expert knowledge. Several high-performance liquid chromatography (HPLC) methods for the quantification of sialic acid [19,20] and monosaccharides [21–23] have been reported. Anion-exchange chromatography with a spectrophotometric detector is reliable and easily implemented, but requires a long analytical time and affords low sensitivity. The HPLC method using chemically bonded silica gel column [24] is rapid, but relatively insensitive and does not stand up to long use. The fluorescent pyridylamino derivative method [25,26] takes 2 days for analysis and involves laborious procedures. Recently, the method of anion-exchange chromatography followed by pulse-amperometric detection has been developed [27–29]. This method has the advantage of sensitivity, but in the case of biological samples, such as AAG, many interfering peaks were observed.

We therefore developed a simple and rapid purification method for AAG by using ion-exchange and hydroxyapatite columns. We also developed an improved method for the determination of N-acetylneuraminic acid (NeuAc) and monosaccharides in the carbohydrate moiety of AAG (fucose, N-acetylglucosamine, galactose, mannose) by using an ion-exchange column (for cleaning up the injected sample) followed by pulse-amperometric detection. Then we examined the differences in the concentration of the carbohydrate moiety of the purified plasma AAG between healthy subjects and patients with renal insufficiency.

2. Experimental

2.1. Materials and sample preparation

The plasma used in this study was obtained from 8 healthy subjects (4 men and 4 women, age 65.8 ± 12.5, mean ± S.D.) and 6 patients (5 men and 1 woman, age 60.2 ± 7.2) with renal insufficiency (creatinine clearance <50 ml/min). The plasma samples were frozen at −80°C until use. Human AAG (Lot. 57F-9319), fucose and N-acetylglucosamine were purchased from Sigma (St. Louis, MO, USA). NeuAc, galactose and mannose were from Wako Pure Chemical (Osaka, Japan). All other reagents were of the highest grade available and used without further purification. A TOYOPAK DEAE-M anion-exchange column (gel volume 1.0 ml), IC-SP cation-exchange column (gel volume 1.0 ml; Tosoh, Tokyo, Japan), hydroxyapatite column (PC-pure, packed volume 0.4 g; Moritex, Tokyo, Japan), and Ampure SA column (55×15 mm I.D.) for desalting (Amasham Japan, Tokyo, Japan) were washed with 5 ml of distilled water before use.

2.2. Isolation of AAG

A 1-ml aliquot of plasma was diluted to 10 ml with 0.01 *M* citrate-phosphate buffer (pH 4.0; buffer A), and then the mixture was loaded onto a DEAE-M column. After washing the column with 10 ml of buffer A, the AAG was eluted with 3 ml of 0.1 *M* citrate-phosphate buffer (pH 4.0), and then the eluate was loaded onto an IC-SP column. The eluate was directly loaded onto an Ampure SA column. Desalted eluate was loaded onto a PC-pure column. After washing the column with 10 ml of distilled water, the AAG was eluted with 3 ml of buffer A, and then the eluate was desalted by an Ampure SA column and freeze-dried. All elution procedures were run at 4°C.

2.3. Neuraminidase treatment

AAG in 0.01 *M* citrate-phosphate buffer (pH 5.0) was incubated with neuraminidase (0.05 units mg^{-1} of AAG) in a shaking bath at 37°C. At stated time intervals, aliquots of the incubated solution were cooled at 0°C.

2.4. Electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a slab separating gel 1.0 mm thick containing 12% acrylamide. The protein samples were incubated with 0.9% mercaptoethanol for 10 min in a boiling water-bath.

2.5. Hydrolysis of AAG

2.5.1. Analysis of monosaccharides

Acid hydrolysis of carbohydrates was carried out by the method of Suzuki et al. [26] and Hardy et al. [27]. A 0.1-ml sample of AAG (0.2–0.8 mg/ml AAG) and 0.25 ml of internal standard (10 μg/ml mannitol) were added to 0.5 ml of 4.0 *M* trifluoroacetic acid (TFA) solution, and then the mixture was heated at 100°C for 4 h in an evacuated sealed tube. After hydrolysis, the solution was evaporated to dryness in vacuo, and the residue was dissolved in 1.0 ml of 0.05 *M* HCl. The solution was loaded onto a DEAE-M column, which had been prewashed with 5.0 ml of 0.05 *M* HCl, to remove interfering compounds. The effluent volume changed depending on the amount of filtrate; therefore, it was then evaporated to dryness, and the residue was dissolved again in 0.5 ml of distilled water. The solution was then injected into the HPLC system.

2.5.2. Analysis of NeuAc

AAG solution and 0.1 ml of internal standard (25 μg/ml N-glycolylneuraminic acid; NeuGc) were added to 0.2 ml of 0.05 *M* HCl, and the mixture was heated at 80°C for 1.0 h. The solution was then passed through an IC-SP column, which had been prewashed with 5.0 ml of 0.05 *M* HCl, to remove interfering compounds. The effluent was directly injected into the HPLC system. The injected volume (30–50 μl) was changed depending on the amount of filtrate.

2.6. Analytical methods

AAG concentration was determined by HPLC as described previously [30]. NeuAc and each monosaccharide concentration were determined as follows. An HPLC system (L-6020; Hitachi, Tokyo, Japan) equipped with an HP1049A electrochemical detector (Hewlett Packard, Waldbronn, Germany) was used. NeuAc separation was achieved on a column (300 × 7.8 mm I.D.) of Excelpak CHA-Ell (Yokogawa, Tokyo, Japan) at a flow-rate of 0.75 ml/min and a column temperature of 50°C. The mobile phase was 1 m*M* H_2SO_4, and the reagent solution in the detection was 0.6 *M* NaOH. Monosaccharide separation was achieved on a column (250 × 4.0 mm I.D.) of Dionex Carbopak PAl (CA, USA) at a flow-rate of 1.0 ml/min and room temperature. The detection of monosaccharides was carried out at an isocratic NaOH concentration of 16 m*M*. The peak was measured by pulse- amperometric detection using a gold working electrode and integrated with a Hitachi D-2500 data processor. The following pulse potentials and durations were used: $E_1 = 0.05$ V ($t_1 = 300$ ms); $E_2 = 0.65$ V ($t_2 = 100$ ms); $E_3 = -0.95$ V ($t_3 = 300$ ms). The response time was set at 8 s.

2.7. Statistical analysis

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

3. Results and discussion

3.1. Purification of human plasma AAG

The purity was checked by a radio-immunodiffusion method and SDS-PAGE. The efficiency of the purification method was monitored using

immunodiffusion. With SDS-PAGE, before the PC-pure column treatment, few bands were found. However, in this proposed method using a combination of ion-exchange and hydroxyapatite columns, both of the purified AAGs from the plasma of healthy subjects and from the patients with renal insufficiency showed a single band with an electrophoretic mobility which was in agreement with that of the Sigma AAG (Fig. 1). After exhaustive desialylation with neuraminidase, the native band disappeared completely, with a slightly lower relative molecular mass. These results strongly suggest that this purification procedure was capable of isolating AAG in an intact form without denaturating the carbohydrate moiety.

The present method for the purification of AAG has several advantages over the previously published techniques. Firstly, the purification of AAG could be done rapidly (less than 1.0 h) without structural degradation. Secondly, the volume of plasma needed for the purification of AAG and for the following determination of monosaccharide was less than 1.0 ml. Thirdly, a very high recovery rate (more than 80%) was obtained.

3.2. Analysis of NeuAc and monosaccharides

Typical chromatograms of NeuAc and monosaccharides obtained from healthy subjects and from patients with renal insufficiency are shown in Figs. 2 and 3, respectively. In this method, the clean-up procedures, by IC-SP column for NeuAc and by DEAE-M column for monosaccharides, are required for the removal of interfering peaks. Standard curves were obtained according to the procedure described in Section 2. A linear relation between the amount of NeuAc or monosaccharides and the peak-height ratio of NeuAc/monosaccharide to internal standard was observed over the concentration range from 5.0 to 100 μg/ml. NeuAc: $y = 0.021x +$

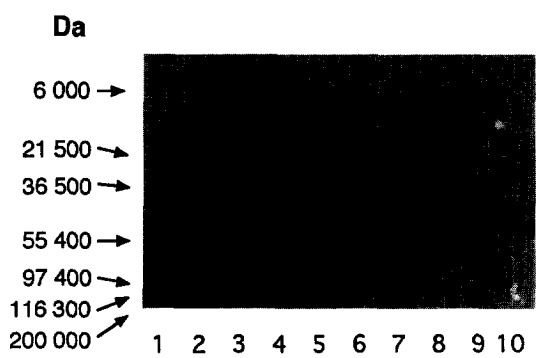

Fig. 1. SDS-polyacrylamide gel electrophoresis of asialo AAG, Sigma AAG and purified AAG from plasma of patients with renal insufficiency and healthy subjects. For each sample: lines 1,2, marker proteins; lines 3,4, purified AAG from healthy subjects and patients with renal insufficiency without hydroxyapatite column, respectively; lines 5,6, purified AAG from healthy subjects and patients with renal insufficiency with the hydroxyapatite column, respectively; lines 7,8, asialo AAG from healthy subjects and patients with renal insufficiency, respectively; lines 9,10, Sigma AAG.

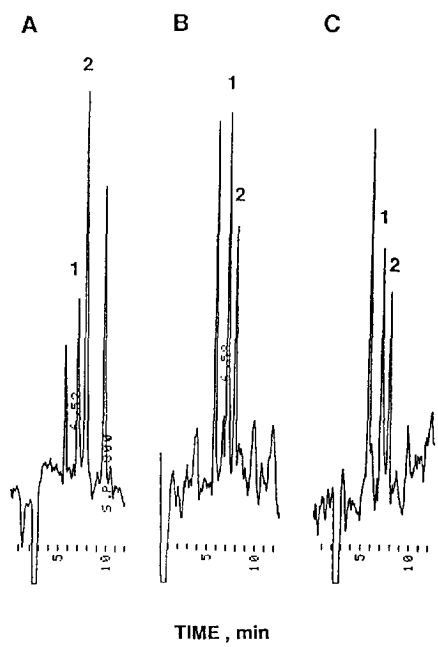

Fig. 2. Representative chromatograms of N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc) after treatment of standard and plasma samples. (A) Standard sample containing 6.25 μg/ml NeuAc, (B) healthy subject, (C) patient with renal insufficiency. Peaks: 1 = NeuGc (internal standard); 2 = NeuAc.

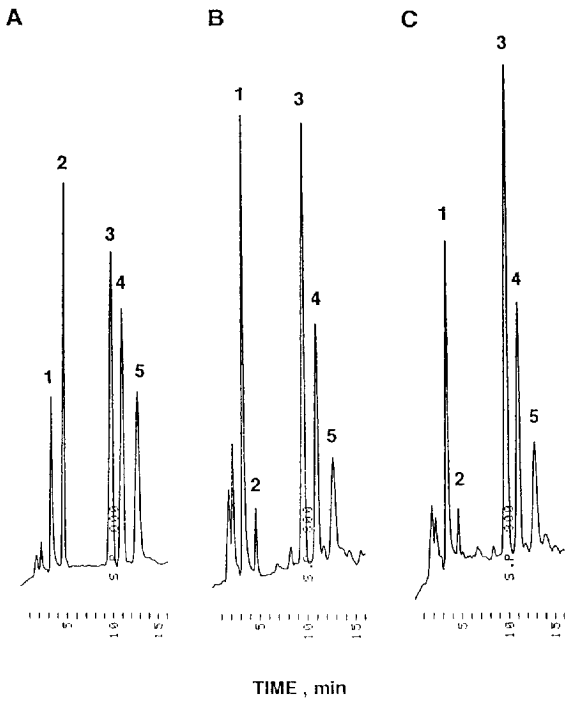

Fig. 3. Representative chromatograms of monosaccharides after treatment of standard and plasma samples. (A) Standard sample containing 5.0 μg/ml of each monosaccharide, (B) healthy subject, (C) patient with renal insufficiency. Peaks: 1 = mannitol (internal standard); 2 = fucose; 3 = N-acetylglucosamine; 4 = galactose; 5 = mannose.

0.008 ($r = 1.000$); Fuc: $y = 0.026x + 0.009$ ($r = 1.000$); GluNAc: $y = 0.018x + 0.027$ ($r = 0.999$); Gal: $y = 0.015x + 0.017$ ($r = 0.999$); Man: $y = 0.010x + 0.027$ ($r = 0.999$). The detection limits for NeuAc and monosaccharide quantification were 2 pmol and 1 pmol ($S/N > 2$), respectively.

3.3. Determination of NeuAc and monosaccharides in AAG from healthy subjects and patients with renal insufficiency

AAG concentration in the serum of patients with renal insufficiency was significantly higher than that in healthy subjects ($p < 0.05$).

The concentration of NeuAc and monosaccharides in the purified plasma AAGs from healthy subjects and patients are shown in Tables 1 and 2, respectively. These results of "normal" AAG are rather low, when compared with those previously reported by Schmit et al. [31,32]. They analyzed the carbohydrate content and composition of an AAG isolated from pooled plasma, and they did not describe the background of the subjects, such as age, sex and racial or ethnic groups. According to a recent report, the carbohydrate structure of some glycoproteins including AAG changed with sex or age [33]. The aim of the present study was to clarify the change of

Table 1
Analysis of N-acetylneuraminic acid (NeuAc) and monosaccharide level in purified α-1-acid glycoprotein (AAG) from plasma of healthy subjects

Subjects	Sex	Age (years)	AAG conc. (mg/ml)	NeuAc (mg/g AAG)	Fuc. (mg/g AAG)	GluNAc (mg/g AAG)	Gal. (mg/g AAG)	Man. (mg/g AAG)
K.J.	M	70	0.99	65.87	5.88	102.06	69.10	45.50
Y.I.	M	60	0.78	77.25	9.66	131.70	83.69	50.67
K.T.	M	68	1.03	61.95	11.12	109.37	76.73	52.74
T.A.	M	46	0.66	88.39	11.17	108.86	73.96	49.70
T.M.	F	60	0.72	89.48	9.88	115.32	78.47	51.68
T.T.	F	84	0.66	93.83	8.93	114.40	78.56	47.29
M.K.	F	84	0.66	97.94	16.18	123.39	80.88	50.03
Y.Y.	F	55	0.78	87.44	5.86	98.97	74.38	45.47
Mean		65.8	0.79	82.77	9.84	113.01	76.97	49.14
S.D.		12.5	0.14	12.25	3.08	10.07	4.23	2.57

Table 2
Analysis of N-acetylneuraminic acid (NeuAc) and monosaccharide level in purified α-1-acid glycoprotein (AAG) from plasma of patients with renal insufficiency

Subjects	Sex	Age (years)	AAG conc. (mg/ml)	NeuAc (mg/g AAG)	Fuc. (mg/g AAG)	GluNAc (mg/g AAG)	Gal. (mg/g AAG)	Man. (mg/g AAG)
S.S.	F	48	1.20	74.87	12.02	137.91	85.84	52.34
T.O.	M	65	0.99	98.52	9.11	124.07	83.53	53.95
T.K.	M	61	0.72	97.54	9.21	151.49	94.92	59.22
T.F.	M	67	1.23	64.56	16.50	139.21	85.74	58.25
I.T.	M	56	0.93	100.97	13.11	136.51	85.77	56.91
K.I.	M	65	0.99	99.31	16.77	123.44	96.85	62.91
Mean		60.2	1.01	89.29	12.79	135.44	88.78	57.26
S.D.		7.2	0.19	15.55	3.37	10.51	5.61	3.80

carbohydrate content and composition of AAG in a disease state such as renal insufficiency. Therefore, the mean age of the healthy subjects in this study was relatively high. We think that patient age and/or racial differences are the reasons for the contradiction between the results of Schmit et al. and our data. Furthermore, Fournet et al. [34] and Yoshima et al. [35] reported that each glycosylation site of AAG possesses a different carbohydrate structure. Schmit et al. reported that the molar Gal/Man ratio of the carbohydrate moiety of AAG is in the range of 0.7–2.5 [36]. Collectively, these results highly suggest that the carbohydrate composition of AAG from healthy subjects is variable. In the subjects with renal insufficiency, on the other hand, the mean concentrations of N-acetylglucosamine, galactose and mannose were significantly higher than those in the healthy subjects, whereas the values of NeuAc and fucose in the patients were almost the same as those of the healthy subjects (Fig. 4). Although the mechanism and the biological significance are still unclear, an alteration of the carbohydrate moiety of AAG in several disease states has been reported [10,12,13]. In particular, Smith et al. [11] have reported that a noticeable difference in the glycosylation pattern was founded by the rheumatoid AAG sample which contains more bi- and tetra-antennary chains than in the AAG from normal plasma. These changes of the carbohydrate moiety in the disease state were compatible with our results.

The change of the carbohydrate moiety in the AAG of several disease states and its physiological meaning should be further examined. The present method, that is, the rapid purification of AAG and the following sensitive determination of monosaccharides, would be useful for these examinations.

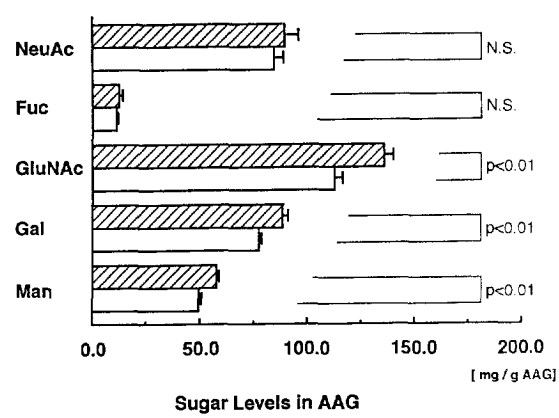

Fig. 4. Comparison of N-acetylneuraminic acid (NeuAc) and monosaccharide level in purified α-1-acid glycoprotein (AAG) between patients with renal insufficiency (hatched columns) and healthy subjects (blank columns). Columns represent mean values with SEM; $p < 0.01$ indicates a significant difference between the group with renal insufficiency and the healthy group.

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