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Determination of Neutral and Amino Sugars in Glycoproteins by Gas Chromatography

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A gas chromatographic method for the determination of neutral and amino sugars in glycoproteins was developed by using trifluoroacetyl derivatives of sugar alcohols and by selecting trifluoroacetic acid as acid catalyst for hydrolysis. The method is as follows: As the hydrolytic condition, 2.5 N TFA, 100°, 7 hr was common to obtain excellent recovery of neutral and amino sugars in many glycoproteins. In the case of having enough sample, it was recommendable to select 4 and 7 hr for hydrolysis of neutral sugars and 7 and 10 hr for amino sugars. In sample preparation for gas chromatography, sugars were reduced with sodium borohydride to corresponding sugar alcohols and trifluoroacetylated with trifluoroacetic anhydride in ethyl acetate. This method was further improved for micro-analysis of sugars in glycoproteins, taking full advantage of the sensitivity of an electron capture detector (ECD) to trifluoroacetyl derivatives. Consequently, it was suggested to be able to analyze constituent neutral and amino sugars in microgram amounts of glycoproteins.

Gas chromatography is one of the most successful methods for the identification and quantification of sugars in a mixture. In the previous reports, we described the gas chromatographic methods for quantitative analysis of aldoses[2)] and amino sugars[3)] with a hydrogen flame ionization detector (HFID), which involved the reduction of sugars to corresponding sugar alcohols with sodium borohydride followed by the trifluoroacetylation of sugar alcohols with trifluoroacetic anhydride (TFAA) in ethyl acetate (AcOEt).

In this work, we examined the application of these methods to analysis of constituent sugars in glycoproteins. Especially in hydrolysis of glycoprotein, we selected trifluoroacetic acid (TFA) as acid catalyst and examined its hydrolytic condition for various materials, because it was difficult to avoid loss of released sugars in hydrolysis with mineral acids. Consequently we established the procedure for the quantitative analysis of constituent neutral and amino sugars of glycoproteins by gas chromatography. In addition, we further tried to develop this method to micro-analysis of sugars in glycoproteins, taking full advantage of high sensitivity of an electron capture detector (ECD) to trifluoroacetyl derivatives.

Experimental

Materials——Ethyl acetate (GR: Kanto Chemical Co., Ltd.), trifluoroacetic anhydride (GR: Tokyo Kasei Kogyo Co., Ltd.), sodium borohydride (GR: Merck AG) and trifluoroacetic acid (GR: Tokyo Kasei Kogyo Co., Ltd.) were used directly. Amberlite CG-120 (H^+) resin (type 1) was conditioned by washing with 2 N HCl followed by repeated washing distilled water. Amberlite CG-4B (CH_3COO^-) resin (type 1) was conditioned by washing with 2 N CH_3COOH followed by repeated washing with distilled water. QAE-Sephadex (borate form) was conditioned by washing with saturated solution of $K_2B_4O_7 \cdot 5H_2O$ followed by repeated washing with distilled water. Aldoses, alditols and hexosamines were the commercial samples. N-Acetylglucosaminides were kindly gifted by Dr. Kinoshita, Faculty of Pharmaceutical Sciences, Showa University. Crystalline ovalbumin (recrystallized 5 times) was from Sigma Chemical Co. Blood group active glycoproteins (A and B type) were originally isolated and purified from human semen.[4)]

Gas Chromatography——The instruments were Simadzu Model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector (HFID) and Hitachi 023 gas chromatograph equipped with an electron

1) Location: *Hongo, Bunkyo-ku, Tokyo.*
2) T. Imanari, Y. Arakawa, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1967 (1969).
3) Z. Tamura, T. Imanari, and Y. Arakawa, *Chem. Pharm. Bull.* (Tokyo), **16**, 1864 (1968).
4) In preparation.

capture detector (ECD). A glass tube (180 cm × 4 mm i.d.) was packed with 2% GE XF-1105 on a support of Gas-Chrom P (60—80 mesh). Other condition weres shown in figures.

Preparation of Samples——An outline of the sample preparation for analysis of sugars in glycoproteins is shown in Charts 1 and 2. In most experiments for analysis of neutral sugars and amino sugars, 1—5 mg of glycoproteins was hydrolyzed in 2 ml of 2.5 N TFA at 100° in an evacuated sealed tube. In case of aqueous sample, 1 ml of 5 N TFA was added to 1 ml of sample solution in the tube. The time for hydrolysis was 7 hr usually. After hydrolysis, the tube was cut and a suitable amount of the aqueous solution of internal standard was added to the materials in the tube. The mixture was washed out with an equal volume of water into a 5 ml pear-shaped flask. The solution was evaporated to dryness under reduced pressure at 40° and the residue was dissolved in a small amount (0.5—1 ml) of water. The solution was then passed through an Amberlite CG-120 (H^+) column (0.8 × 5 cm).

a) For analysis of neutral sugars (aldoses), the filtrate was passed through an Amberlite CG-4B (CH_3COO^-) column (0.8 × 5 cm) to remove some remaining anions. The effluent was then evaporated to dryness and the residue was dissolved again in 0.5 ml of water. To the aqueous sample solution, 0.5 ml of 1% $NaBH_4$ in water was added dropwise. The mixture was allowed to stand for 30 min at room temperature and then the excess of $NaBH_4$ was destroyed by addition of 10% CH_3COOH until foaming was stopped. The sample solution was passed through an Amberlite CG-120 (H^+) column (0.8 × 5 cm) followed by washing with 1 ml of distilled water. The effluent was collected to a 5 ml pear-shaped flask and then evaporated under reduced pressure at 40°. To the residue, 2 ml of absolute methanol was added and the solution was evaporated to dryness at room temperature. The last procedure, by which the borate was removed as methyl borate, was repeated twice. The alditols produced were dried in a vacuum desiccator.

b) For hexosamine analysis, the column of Amberlite CG-120 (H^+) was eluted with 5 ml of 1 N HCl and the eluate was evaporated to dryness. The residue was dissolved in 0.5 ml of distilled water, and 0.5 ml of 1% $NaBH_4$ solution was added dropwise. The mixture was allowed to stand for 30 min at room temperature, and the excess of $NaBH_4$ was destroyed by the addition of drops of conc. HCl–MeOH (1: 23). The solution was evaporated to dryness and then 2 ml of MeOH was added to the residue and boric acid was removed as methyl borate by evaporation. The residue was further dissolved in 1 ml of MeOH and stood for several minutes. The supernatant was transferred to the other flask and the solution was evaporated to dryness (In this procedure, pretty amounts of NaCl was removed.). The residue was dissolved in 0.5 ml of water and passed through a QAE-Sephadex column (borate form) (0.8 × 5 cm) and the column was washed with 10 ml of water. The amino alcohols were eluted with 4 ml of conc. HCl–MeOH (1: 23) and the elute was evaporated to dryness. The boric acid was removed as described above, and the amino alcohols were dried in a vaccum desicuator.

c) Trifluoroacetylation: The dried materials which were obtained by the procedures a) and b) were finally trifluoroacetylated with 0.1 ml of AcOEt[5] and 0.1 ml of TFAA at 0°, and then the solution was allowed to stand at room temperature for about 30 min. One to two μl of the reaction mixture was injected directly to the gas chromatograph under suitable conditions.

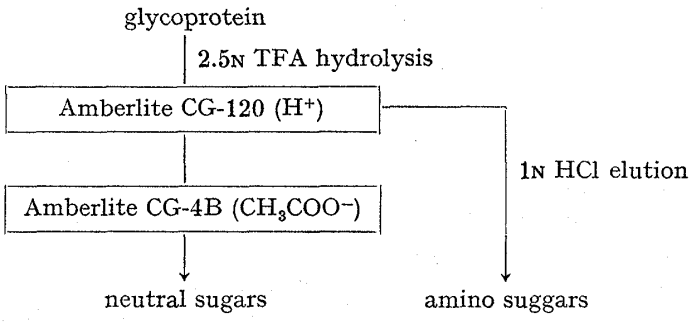

Chart 1. Purification of Sugars

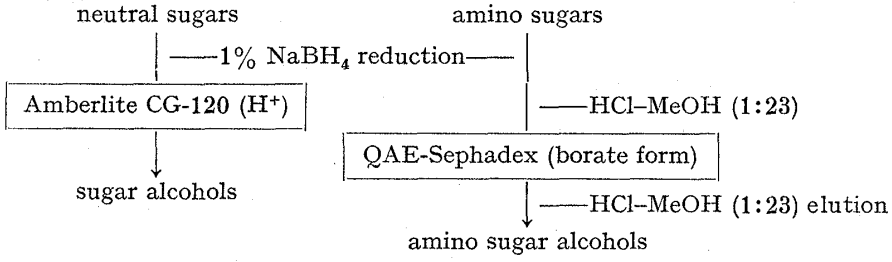

Chart 2. Reduction of Sugars

5) As an alternative to AcOEt, N,N-dimethylformamide (DMF) enhances the reaction to complete it within 5 min at room temperature, although the more pronounced tailing due to the solvent is observed.

Results and Discussion

The recovery of monosaccharides from authentic mixture solution was at first examined through the analytical procedure (Chart 1) except hydrolysis step and the recovery percents of them were about 98%.

Therefore if the suitable hydrolytic condition producing no destruction is selected and the internal standard is added before or just after hydrolysis, the analysis of sugars from glycoproteins will be complete.

Basic Studies of Hydrolysis with TFA

After the acid hydrolysis of a glycoprotein, the acid must be first removed by evaporation or passing through the ion-exchange resin. Although evaporation is the rapidest method in the treatments, it is impossible to remove mineral acid without destruction of sugars. But TFA is volatile (bp 73°) and is easily removed by evaporation.

Then we examined the stability of sugars during evaporation of TFA. The results showed that all the sugars were stable at TFA concentration of 2.5N, 5N or 10N under reduced pressure at 40°.

Hydrolysis of oligosaccharides in glycoproteins by mineral acids contains many problems concerning the destruction of sugars and the resistance of some glycosidic bonds (*e.g.*, bonds involving N-Acetylhexosamines). Particularly, destruction of sugars is occasionally enhanced by the presence of amino acids. With this point of view, authentic sugars were heated at 100° for 4 hr in the presence of some amino acids, with increase in the concentration of TFA from 0.5N to 10N. As the results, all the sugars were quite stable at less than 2.5N TFA and remarkable influence by the presence of amino acids was not seen (Fig. 1). Next, the stability of sugars on heating under various duration with 2.5N TFA was examined. Both neutral and amino sugars were stable within 8 hr and the presence of amino acids did not cause any significant loss of sugars (Fig. 2).

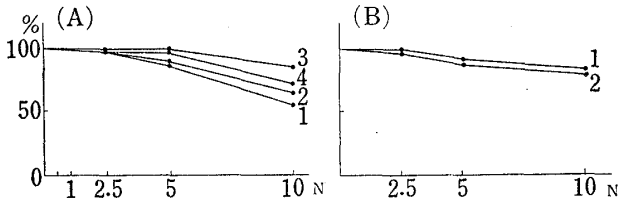

Fig. 1. Effect of TFA Concentration on Recovery of Neutral (A) and Amino Sugars (B)

Standard solutions of four neutral sugars (fucose, 25 μg; mannose, 50 μg; glucose, 100 μg; galactose, 100 μg) or two amino sugars (N-Ac-glucosamine, 100 μg; N-Ac-galactosamine, 100 μg) were heated at 100° for 4 hr under the various concentrations of TFA in the presence of amino acids (*e.g.*, aspartic acid, 200 μg; threonine, 100 μg; serine, 150 μg). The sugars were determined by the gas chromatography under the addition of internal standards (xylose, 50 μg or mannosamine, 150 μg).
gas chromatographic conditions: 2% XF-1105, 180 cm × 4 mm i.d., 140° or 180°, N_2 70 ml/min
(A) 1: fucose 2: mannose 3: glucose 4: galactose
(B) 1: glucosamine 2: galactosamine

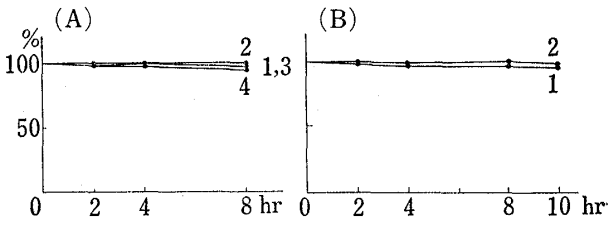

Fig. 2. Effect of Heating Time on Recovery of Neutral (A) and Amino Sugars (B)

The same mixture solutions as in Fig. 1 were heated at 100° with 2.5 N TFA under varing times. The sugars were determined by same method as in Fig. 1.
(A) 1: fucose 2: mannose 3: glucose 4: galactose
(B) 1: glucosamine 2: galactosamine

Under the selected acidity of 2.5N TFA, the hydrolysis of cellobiose, which may be the most stable among neutral disaccharides, was completed after 4 hr with no destruction during the subsequent 2 hr (Fig. 3). N-Acetyl glucosaminides which were more resistant to acid hydrolysis gave a maximum level of glucosamine at 8 hr under the same condition (Fig. 4).

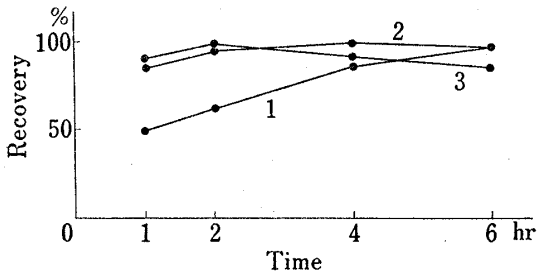

Fig. 3. Hydrolysis of Cellobiose by TFA

Cellobiose (100 μg) was hydrolyzed at 100° under various concentrations of TFA and duration, and subjected to the gas chromatography using 50 μg of xylose as internal standard.
1: 0.5 N TFA 2: 2.5 N TFA 3: 5 N TFA

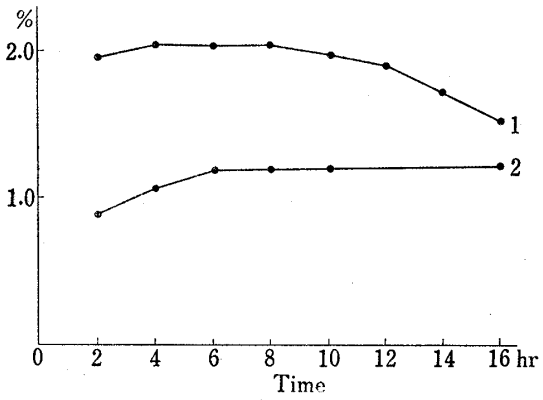

Fig. 5. Released Mannose and Glucosamine (percent by weight) after Hydrolysis of Ovalbumin under Various Times

1: mannose 2: glucosamine

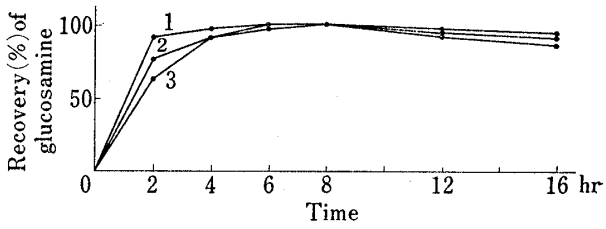

Fig. 4. Hydrolysis of N-Acetyl Hexosaminides with TFA

Three N-acetyl hexosaminides (200 μg each) were hydrolyzed at 100° with 2.5 N TFA under varing duration and subjected to the gas chromatography using 300 μg of mannosamine as internal standard.
1: methyl NAc-α-D-glucosaminide
2: chloroethyl NAc-α-D-glucosaminide
3: chloroethyl NAc-β-D-glucosaminide

TABLE I. Mannose Content of Ovalbumin

Method	Mannose content (%)
Gas Chromatography	
Hydrolyzed with 0.25 N H_2SO_4-resin[a] (heated for 24 hr at 100°)	1.73
Hydrolyzed with 2 N HCl (heated for 2 hr at 100°)	1.76
Hydrolyzed with 2.5 N TFA-resin (heated for 4 hr at 100°)	1.80
Hydrolyzed with 2.5 N TFA (heated for 4 hr at 100°)	2.06
Value from literature[6]	2.0

a) J.H. Kim, B. Shome, T. Liao, and J.G. Pierce, *Anal. Biochem.*, **20**, 258 (1967)

The Release of Monosaccharides from Glycoproteins during TFA Hydrolysis

Ovalbumin (10 mg) was hydrolyzed in 2 ml of 2.5N TFA at 100° and subjected to the full analytical technique (Charts 1, 2) under the addition of internal standards (xylose, 100 μg; mannosamine, 300 μg).

As shown in Fig. 5, mannose was completely released after 4 hr with no destruction within the subsequent 4 hr. On the other hand, the amounts of glucosamine reached a maximum at 6—8 hr followed by no destruction for 10 hr. Table I shows the values of mannose content of ovalbumin obtained by this and other hydrolysis methods. The content of mannose corresponding to 2.06% of ovalbumin, together with the glucosamine content, 1.2% (Fig. 5), agrees well with the value from literature.[6]

From these results, it was found that 2.5N TFA, 100°, 7 hr as hydrolytic condition made it possible to analyze neutral and amino sugars in ovalbumin simultaneously. Moreover, this method was used to the analysis of β-glucuronidase[7,8] and suitable hydrolytic conditions for these materials were almost the same as those of ovalbumin (Table II).

However, it is recommendable to confirm the value by means of selecting 4 and 7 hr for analysis of neutral sugars and 7 and 10 hr for amino sugars in the case of having enough sample.

6) A. Gottshark (ed.), "Glycoproteins," Elsevier Publishing Company, Amsterdam-London-New York, 1966, p. 304.
7) M. Himeno, T. Hashiguchi, and K. Kato, *J. Biochem.*, **76**, 1243 (1974).
8) M. Himeno, H. Ohhara, Y. Arakawa, and K. Kato, *J. Biochem.*, **77**, 427 (1975).

Table II. Hexose and Hexosamine Content (%) of β-Glucuronidase[7]

Hydrolysis condition	2.5 N TFA 4 hr	2.5 N TFA 7 hr	2.5 N TFA 10 hr
Hexose			
L-Fucose	trace	trace	trace
D-Mannose	2.86	2.82	2.75
D-Glucose	0.45	0.43	0.42
D-Galactose	0.48	0.49	0.45
Hexosamine			
D-Glucosamine	1.72	2.01	2.07

β-Glucuronidase (5.046 mg) was hydrolyzed at 100° and subjected to the gas chromatography using 101.7 μg of 3—O-methyl-D-glucose and 426.2 μg of mannosamine as internal standard.

Micro-analysis of Neutral and Amino Sugars

For the investigation of trace amounts of sugars in biological materials, the development of micro-analytical method has been ultimately required. We have already reported the micro-analysis of sugars by gas chromatography with ECD using TFA derivatives.[9] In this experiment, trifluoroacetylation of sugar alcohols was conducted as follows: To 10—500 ng of sugar alcohols, 10 μl of CH_3CN and 2 μl of TFAA were added at 0°. The mixture was kept for 30 min with occassional shaking at room temperature and was added 0.5—1.0 ml of CH_3CN and 1 μl aliquot of the solution was injected into the gas chromatograph.

The satisfactory separation of neutral and amino sugars was achieved on 2% XF-1105 column in 20 min under the same condition as gas chromatography with HFID.

Further, for application of this gas chromatographic method to analysis of constituent sugars of glycoproteins, sample preparation procedure (Charts 1 and 2) was scaled down as follows: 1—10 μg of glycoprotein, 0.2 ml of 2.5N TFA in hydrolysis, Amberlite CG-120 (H^+) column (0.4×5 cm) and Amberlite CG-4B (CH_3COO^-) column (0.4×5 cm) in purification of sugars, 0.1 ml

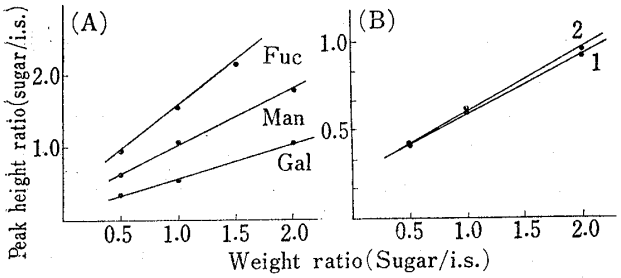

Fig. 6. Calibration Curves for Neutral (A) and Amino Sugars (B)

Xylose (100 ng) and mannosamine (500 ng) were used as internal standard for neutral and amino sugars respectively.
1: $GluNH_2$ 2: $GalNH_2$

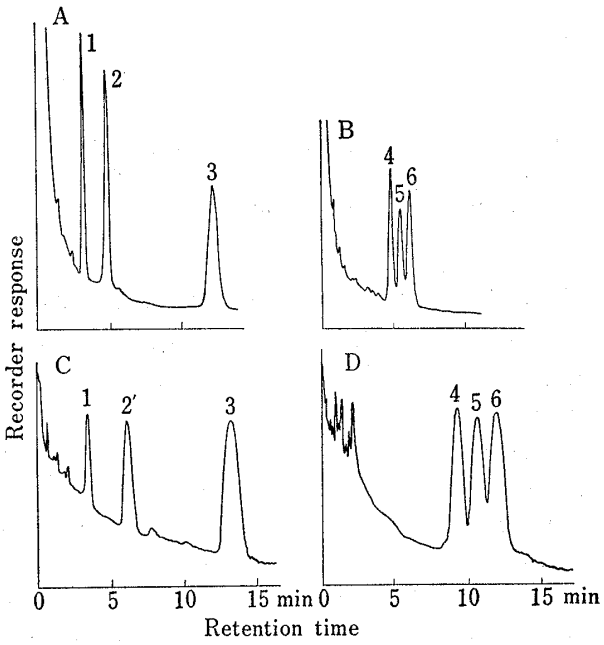

Fig. 7. Gas Chromatograms of Neutral and Amino Sugars in B Type Blood Group Active Glycoprotein

A: neutral sugars (HFID) B: amino sugars (HFID)
C: neutral sugars (ECD) D: amino sugars (ECD)
peak: 1. fucose 2. arabinose (i. s.) 2′. xylose (i. s.)
3. galactose 4. glucosamine 5. galactosamine
6. mannosamine (i. s.)
column: 2% XF-1105 (on Gas-Chrom P) 1.8 m×4 mm i. d.,
column temperature: 140° (HFID, ECD method) for neutral sugars 180° (HFID method), 175° (ECD method) for amino sugars
carrier gas: N_2 65 ml/min

9) Z. Tamura and T. Imanari, *Chem. Pharm. Bull.* (Tokyo), **15**, 246 (1967).

of 0.5% $NaBH_4$ in reduction, 5% CH_3COOH in destroying of excess $NaBH_4$ 1 ml of MeOH in removing boric acid, 1 ml of 1N HCl in elution of amino sugars, 0.1 ml of MeOH in desalting, QAE-Sephadex (borate form) column (0.4×5 cm), 2 ml of H_2O and 2 ml of conc. HCl–MeOH (1:23) in washing and elution of amino alcohols.

Calibration curves of neutral and amino sugars in this micro-analysis are shown in Fig. 6. The recoveries of pure monosaccharides from the authentic mixture solution through the whole analytical procedure were 90—95%.

Both this micro-analytical procedure (ECD method) and usual procedure (HFID method) were applied to the analysis of constituent sugars in A and B type blood group active glycoproteins from human semen which were originally purified in our laboratory.[4] Comparison of the results were shown in Table III and Fig. 7 and the good agreement was obtained with an error of about 10%.

Table III. Sugar Content (%) of Blood Group Active Glycoprotein in Human Semen Determined by ECD and HFID Methods

	A type		B type	
	ECD	HFID	ECD	HFID
Fucose	3.9	4.3	4.7	4.6
Galactose	10.0	9.6	13.5	12.8
Glucosamine	4.5	4.4	10.1	8.7
Galactosamine	6.8	6.0	8.2	7.7

Ten μg of the glycoprotein was subjected to ECD method and 1 mg was subjected to HFID method. Gas chromatographic conditions are described in Fig. 9.
Internal standard: xylose (0.5 μg), mannosamine (1.02 μg) for ECD method, arabinose (51.3 μg), mannosamine (100.8 μg) for HFID method

This experiment suggests the possibility of analyzing constituent neutral and amino sugars in microgram amounts of glycoproteins.

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