Journal of Chromatography, 226 (1981) 341—350
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1019

ANALYSIS OF THE MONOSACCHARIDE COMPOSITIONS OF TOTAL NON-DIALYZABLE URINARY GLYCOCONJUGATES BY THE DITHIOACETAL METHOD

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(Received April 21st, 1981)

SUMMARY

The component aldoses, uronic acid, and hexosamines in the total non-dialyzable urinary glycoconjugates were determined by the dithioacetal method, and normal levels of these monosaccharides are presented. The molar ratios of fucose/galactose, glucuronic acid/galactose, and galactosamine/glucosamine for cancer patients were lower, and that of mannose/galactose was higher, than normal values.

INTRODUCTION

Human urine contains a variety of carbohydrates, including mono- and oligosaccharides, mucopolysaccharides, glycolipids, and glycoproteins, and there have been a number of studies on their structures, biosynthesis, and metabolism. However, analytical study from the clinical viewpoint seems insufficient, except for the major carbohydrates such as glucose in diabetes mellitus and mucopolysaccharides in some hereditary diseases. Under these circumstances our

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project aims at the establishment of correlation between microheterogeneity of carbohydrate chains in these glycoconjugates and pathological conditions. In previous papers we reported a general method for the rapid analysis of monosaccharides [1, 2] and products of periodate oxidation of carbohydrates [3, 4] as trimethylsilylated dithioacetals. This paper discusses the problems in applying this method to the analysis of monosaccharide compositions in total non-dialyzable urinary glycoconjugates and presents preliminary results obtained for urines from normal subjects and cancer patients.

MATERIALS AND METHODS

Chemicals

Ethanethiol and chlorotrimethylsilane were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Trifluoroacetic acid (TFA) and hexamethyldisilazane were from Wako Pure Chemicals (Osaka, Japan). All other chemicals, solvents, and samples of carbohydrates were of the highest grade commercially available.

Urine samples

Urine samples were collected before breakfast from sixteen normal volunteers and fifteen cancer patients. The patients include those with carcinomas of the lung (3), stomach (2), colon (3), ovary (2), breast (3), liver (1), and pancreas (1), where the numbers in parentheses are those of patients. No cases of metastasis were included. Age distribution for the normal volunteers were as follows; twenties (4), thirties (3), forties (2), fifties (2), sixties (2), seventies (2), and eighties (1). A 50-ml portion of each urine sample was dialyzed in a Visking tube for 24 h against running water. The non-dialyzable fraction was lyophilized and a part of the residue was subjected to component analysis.

Apparatus

Gas chromatography was performed on a Shimadzu 4BMPF instrument equipped with a flame ionization detector. A Scott capillary column (50 m × 0.28 mm I.D.) coated with silicone SF-96 was used at 225°C, and the flow-rate of the carrier (nitrogen) was controlled at 1 ml/min by the use of a 100:1 splitter. Scavenger gas (nitrogen) was continuously mixed with the eluates, and the mixtures were introduced into the detector. Peaks were integrated by a Shimadzu E1A integrator.

Hydrolysis of total non-dialyzable urinary glycoconjugates

For the analysis of the component aldoses and uronic acid, the lyophilized non-dialyzable fraction (ca. 1 mg) of a urine sample was weighed into a small ampoule, to which was added a 2 *M* solution of TFA (200 μl). The ampoule was flushed with nitrogen for a few minutes, sealed, and heated for 6 h at 100°C. After cooling, the ampoule was opened, and the hydrolysate was evaporated to dryness under reduced pressure in a desiccator containing pellets of sodium hydroxide.

For the analysis of the component hexosamines the lyophilized non-dialyz-

able fraction (ca. 1 mg) was hydrolyzed similarly in 4 *M* hydrochloric acid (200 μl) for 6 h at 100°C, and the hydrolysate was worked up in the same manner as described for the TFA hydrolysate.

Analysis of the component monosaccharides

The procedure for the analysis of the component aldoses and uronic acid [1] is schematically shown in Fig. 1, together with that for hydrolysis. An aqueous solution (100 μl) of 10^{-3} *M* 3-O-methylglucose (internal standard) was added to each hydrolysate, and the insoluble materials were centrifuged off. The

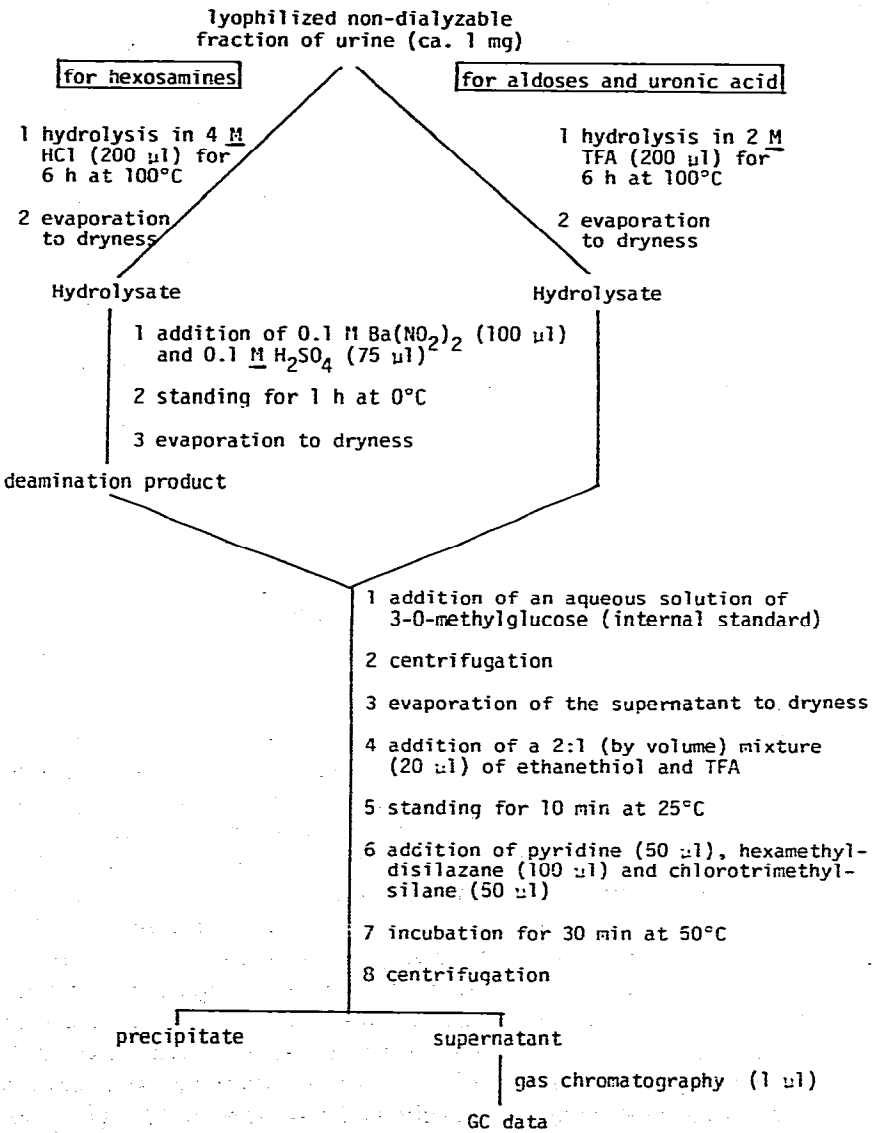

Fig. 1. Procedure for the determination of the component monosaccharides in total non-dialyzable urinary glycoconjugates.

supernatant was transferred with a small volume of washing fluid to a reaction tube (5 cm × 5 mm I.D.) carrying a polyethylene stopper, and evaporated to dryness under reduced pressure in a desiccator containing sodium hydroxide. To the residue from the TFA hydrolysate was added a 2:1 (by volume) mixture (20 μl) of ethanethiol and TFA, and the mixture was kept for 10 min at 25°C with constant swirling. Pyridine (50 μl), hexamethyldisilazane (100 μl), and chlorotrimethylsilane (50 μl) were added in this order, and the mixture was incubated for 30 min at 50°C with occasional shaking. By these sequential treatments, the component aldoses and uronic acid, as well as the internal standard, were converted quantitatively to their trimethylsilylated diethyldithioacetals. The reaction mixture was centrifuged, and a 1-μl sample of the supernatant was injected into the gas chromatography column. The amounts of the component aldoses and uronic acid were determined by comparing their peak areas with those obtained for a standard mixture of authentic monosaccharides under the same conditions.

The component hexosamines were analyzed by another reported procedure [2]. The hydrochloric acid hydrolysate was deaminated with 0.1 *M* barium nitrite (100 μl) and 0.1 *M* sulfuric acid (75 μl) for 1 h at 0°C. The component hexosamines were converted to the corresponding 2,5-anhydrohexoses by this treatment. The mixture was evaporated to dryness under reduced pressure, and the residue was subjected to sequential derivatization of mercaptalation and trimethylsilylation, in the same manner as described above. The amounts of the component hexosamines were calculated similarly by comparing the peak areas with those of a standard mixture.

RESULTS AND DISCUSSION

Gas chromatograms

The procedure used in this work allowed the simultaneous determination of aldoses and uronic acids in ca. 2 h, including the derivatization processes, giving a single peak for each standard sugar. Fig. 2a shows a typical example of a gas chromatogram obtained for the component aldoses and uronic acid of the glycoconjugates in a normal urine sample. Peaks 2, 4, 7, 8, and 9 were assignable to xylose, fucose, glucose, mannose, and galactose, respectively, as the aldose components, and peak 6 to glucuronic acid as the only uronic acid component. The non-dialyzable fraction of urine contains glycoproteins and mucopolysaccharides as the major glycoconjugates, together with small amounts of higher heterooligosaccharides. On the basis of accumulated knowledge of the chemical composition of glycoconjugates, fucose, mannose, and galactose found in the above chromatogram were undoubtedly the component monosaccharides in glycoproteins and heterooligosaccharides, whereas xylose and glucuronic acid (both in part) were contained in mucopolysaccharides. Glucuronic acid could also be freed from its conjugates with various phenolic metabolites. Although human urine contains free glucose, especially in large quantity in diabetes mellitus, peak 7 in this chromatogram was exclusively of the bound glucose, as the urine sample was dialyzed prior to component analysis. It is also apparent that the glucose detected was not an artifact arising from dialysis in a cellophane tube, because the blank test using no urine sample gave no trace of glucose. It

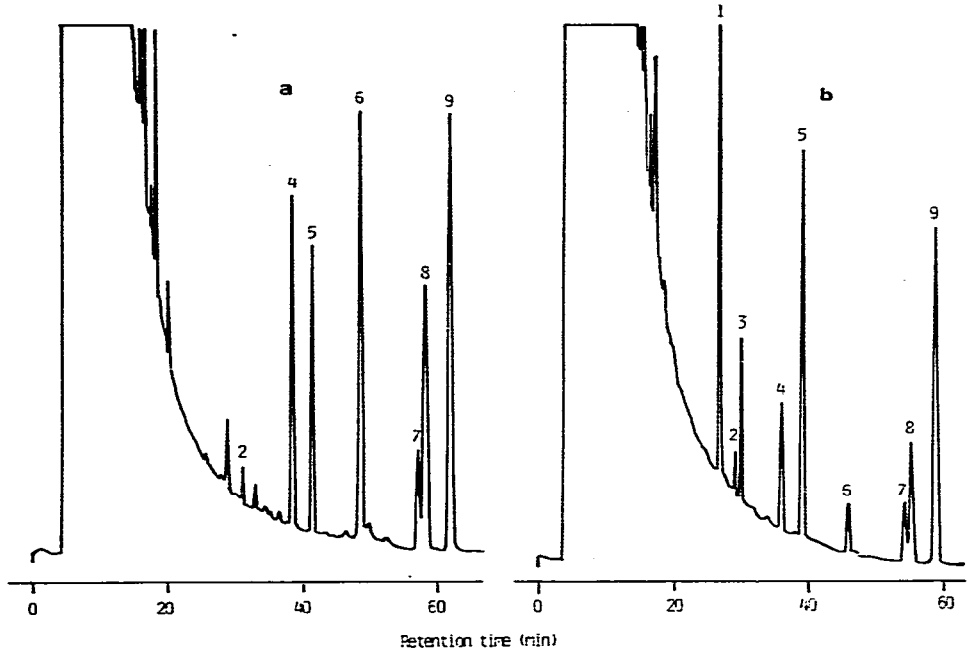

Fig. 2. Typical gas chromatogram obtained for (a) the component aldoses and uronic acid and (b) the component hexosamines in the total non-dialyzable glycoconjugates from a normal urine sample. The component aldoses and uronic acid were analysed as their trimethylsilylated diethyldithioacetals. The component hexosamines were selectively converted to their 2,5-anhydrohexoses with nitrous acid and analysed as the trimethylsilylated diethyldithioacetals of the latter, together with the derivatives of the remaining aldoses and uronic acid. Peak assignment: 1 = glucosamine, 2 = xylose, 3 = galactosamine, 4 = fucose, 5 = 3-O-methylglucose (internal standard), 6 = glucuronic acid, 7 = glucose, 8 = mannose, 9 = galactose.

is not certain from where this bound glucose was derived, but at least it was not a constituent of mucopolysaccharides.

Since hexosamines were not derivatized quantitatively under the conditions used for the aldoses and uronic acid, these hydrolysates were converted to the corresponding 2,5-anhydrohexoses and subsequently derivatized to the trimethylsilylated dithioacetals of the latter. Fig. 2b shows the chromatogram for the same urine sample. The peaks for glucosamine (peak 1) and galactosamine (peak 3) appeared in the pentose region, well separated from each other and also from pentoses. Peaks of some aldoses and glucuronic acid were smaller than those in Fig. 2a due to partial decomposition of these sugars with hydrochloric acid. Both hexosamines may be released from all kinds of non-dialyzable glycoconjugates.

The urine samples from cancer patients and other normal volunteers gave similar chromatograms, but the peak intensities varied among subjects.

Conditions for hydrolysis

For the analysis of component aldoses and uronic acid, TFA was used as the catalyst for hydrolysis because this acid minimized non-hydrolytic degradation. In addition, it is easily removable by evaporation under reduced pressure in a desiccator containing alkali. Fig. 3a shows the course of liberation of these component monosaccharides, as hydrolyzed in 2 *M* TFA at 100°C. The amounts of all these monosaccharides increased gradually to reach plateaus at

TABLE I

RECOVERIES OF THE MONOSACCHARIDES ADDED TO THE HYDROLYSATE OF THE NON-DIALYZABLE FRACTION OF A URINE SAMPLE

n = 5 in each experiment.

Monosaccharide	Average amount found in the hydrolysate (nmol/mg)	Experiment 1			Experiment 2		
		Amount of monosaccharide added (nmol/mg)	Average amount found for total monosaccharide (nmol/mg)	Recovery (%)	Amount of monosaccharide added (nmol/mg)	Average amount found for total monosaccharide (nmol/mg)	Recovery (%)
Xylose	11.2	5	16.0	99	10	20.8	94
Fucose	45.8	25	69.8	99	50	93.6	98
Galactose	112.3	50	159.3	98	100	222.6	105
Glucose	24.2	10	31.5	92	20	41.8	95
Mannose	47.8	25	70.5	97	50	100.2	102
Glucuronic acid	379.8	200	550.4	95	400	750.2	96
Galactosamine	43.5	25	64.5	94	50	90.6	97
Glucosamine	120.3	50	171.3	101	100	204.8	93

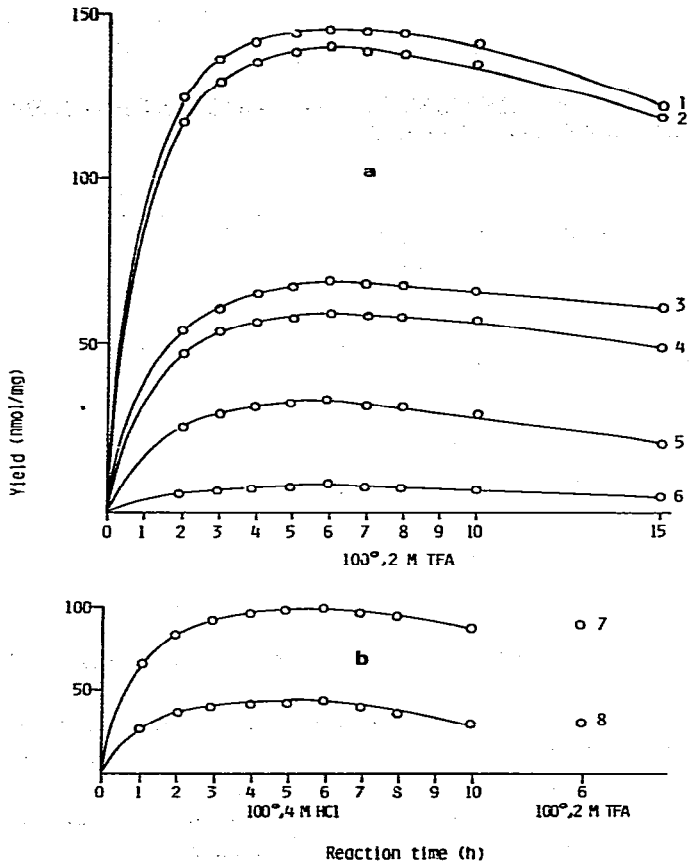

Fig. 3. Course of liberation of (a) the component aldoses and uronic acid and (b) the component hexosamines in the total non-dialyzable glycoconjugates from the same urine sample as used in Fig. 1, as estimated from the peaks of their derivatives. 1 = Glucuronic acid, 2 = galactose, 3 = mannose, 4 = fucose, 5 = glucose, 6 = xylose, 7 = glucosamine, 8 = galactosamine.

ca. 6 h, but prolonged heating resulted in gradual decomposition.

The component hexosamines were hydrolyzed with more difficulty than the aldoses and uronic acid, hence 4 *M* hydrochloric acid was used instead of 2 *M* TFA. The time course shown in Fig. 3b indicates that the appropriate heating time was also 6 h in this case.

Accuracy and precision of component analysis

Correlation of the present method to the conventional alditol acetate method in the determination of the component aldoses and uronic acid was satisfactory, giving the ratios of observed values between 0.94 and 1.05 for all sugars.

Table I shows the recoveries of aldoses added to the hydrolysate of the non-dialyzable fraction of a normal urine sample, as determined by the procedure in Fig. 1. It is indicated that this procedure was satisfactorily accurate. On the other hand Table II gives the coefficients of variation for ten determinations of the component monosaccharides. These data indicate high repeatability of this procedure.

TABLE II

PRECISION OF THE DETERMINATION OF THE COMPONENT MONOSACCHARIDES IN TOTAL NON-DIALYZABLE URINARY GLYCOCONJUGATES

$n = 10$.

Monosaccharide	Amount of monosaccharide (nmol/mg)		Coefficient of variation (%)
	Average	S.D.	
Xylose	10.3	0.37	3.6
Fucose	47.5	1.57	3.3
Galactose	109.7	3.51	3.2
Glucose	23.9	1.12	4.7
Mannose	45.6	2.23	4.9
Glucuronic acid	401.5	20.9	5.2
Galactosamine	42.5	2.17	5.1
Glucosamine	126.2	6.18	4.9

Comparison of the monosaccharide compositions for normal subjects and cancer patients

Table III compares the amounts of individual monosaccharides in total non-dialyzable urinary glycoconjugates for normal subjects and cancer patients. It also gives the molar ratios of these component monosaccharides.

The variation in the amount of xylose was relatively small for normal subjects, but its amout for cancer patients varied over a wide range.

It is noticeable that the average amount of fucose for individual cancer patients was significantly lower than the normal average, with the probability level less than 0.005. The decrease in the molar ratio of fucose to galactose was much more prominent in cancer. Carcinomas of the lung, breast, liver, and pancreas showed an especially marked decrease. The outstanding reduction of the fucose/galactose ratio in cancer is an unexpected finding, as the fucose content in serum glycoprotein was reported to be rather higher in cancer patients [5], suggesting increased activity of GDP-fucosyltransferase in tissues and sera of cancer patients. Therefore, the low level of bound fucose in total non-dialyzable urinary glycoconjugates should be attributed to either an increase in α-fucosidase activity or anomalous production of low-fucose glycoconjugates in secretory organs.

Both mannose and galactose are known to be the major component aldoses of glycoproteins and accordingly hereooligosaccharides derived thereof, but they are not contained in mucopolysaccharides. Their molar ratio is associated with the core structure of the carbohydrate chains in glycoproteins. The results in Table III indicate that the mannose/galactose ratio was varied in a relatively narrow range in both normal subjects and cancer patients, and the average values for the latter were significantly higher than the former. Carcinomas of the stomach, colon, ovary, and liver showed especially high values of the mannose/galactose ratio. The increase in this ratio might be related to the production of a carcinoembryonic antigen [6], which is a kind of glycoprotein

TABLE III

MONOSACCHARIDE COMPOSITIONS OF TOTAL NON-DIALYZABLE URINARY GLYCOCONJUGATES FOR NORMAL SUBJECTS AND CANCER PATIENTS

Monosaccharide composition	Cancer patients (n = 15)			Normal subjects (n = 16)	
	Average	S.D.	p in t-test*	Average	S.D.
Amount of monosaccharide (nmol/mg)					
Xylose (Xyl)	10.5	6.8	<0.4	12.1	2.4
Fucose (Fuc)	31.6	16.6	<0.005	54.4	18.4
Galactose (Gal)	119.1	47.5	<0.6	127.4	29.6
Glucose (Glc)	47.6	46.1	<0.1	25.1	10.5
Mannose (Man)	50.5	19.2	<0.8	48.5	12.7
Glucuronic acid (GlcUA)	184.6	127.3	<0.001	432.3	110.9
Galactosamine ($GalNH_2$)	31.3	14.8	<0.05	41.9	10.8
Glucosamine ($GlcNH_2$)	129.0	66.2	<0.975	128.3	20.9
Molar ratio					
Xyl/Gal	0.10	0.08	NS**	0.10	0.03
Fuc/Gal	0.27	0.07	<0.001	0.42	0.08
Glc/Gal	0.53	0.56	<0.05	0.19	0.06
Man/Gal	0.45	0.10	<0.025	0.38	0.04
GlcUA/Gal	1.52	0.86	<0.001	3.71	1.52
$GalNH_2$/Gal	0.27	0.08	<0.05	0.35	0.13
$GlcNH_2$/Gal	1.11	0.43	<0.9	1.08	0.39
$GalNH_2/GlcNH_2$	0.26	0.09	<0.02	0.33	0.06

*Aspin-Welch t-test was done to judge significant difference between the average of individual cancer patients and that of individual normal subjects.
**Not significant.

having a high mannose/galactose ratio (0.7—0.9) [7].

The amount of glucuronic acid, as well as the glucuronic acid/galactose ratio, was decreased greatly for most samples from cancer patients. Glucuronic acid is the constituent of various glucuronides of phenolic metabolites and mucopolysaccharides, hence the decrement may be assigned to either the former, the latter, or both. However, we could not estimate from the present data alone to what extent each glycoconjugate contributed to the formation of this uronic acid.

Of the two hexosamines found in non-dialyzable glycoconjugates, glucosamine showed no significant alteration of its content in cancer patients. But the average amount of galactosamine for cancer patients was rather smaller than that for normal subjects. The galactosamine/galactose and galactosamine/glucosamine ratios for cancer patients showed a more marked reduction compared to those for normal subjects.

Thus, the gas chromatographic procedure devised on the basis of the dithioacetal method made it possible to determine rapidly the monosaccharides in

non-dialyzable urinary glycoconjugates. The monosaccharide compositions obtained for cancer patients by this procedure are attractive for diagnostic purposes. However, a larger number of samples from both cancer patients and patients with other diseases should be analyzed, before diagnostic evaluation is established. A series of such analyses is now in progress.

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