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Gas chromatographic–mass spectrometric analysis of urinary sugar and sugar alcohols during pregnancy

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Abstract

A refined and simplified method has been developed for the simultaneous analysis of urinary sugar and sugar alcohols after urease treatment by using capillary gas chromatography–mass spectrometry (GC–MS). Since carbohydrate metabolism during pregnancy is considered to be diabetogenic, our interest has been concentrated on understanding the mechanism of the metabolic deviation by assessing the glucose excursion and glucose fluxes. The present study suggests that changes of the levels of glucose, sorbitol, fructose, myo-inositol, and 1,5-anhydro-D-glucitol (1,5-AG) may reflect a mild alteration in carbohydrate metabolism that goes undetected by conventional diabetic indicators.

Keywords: Sugars; Sugar alcohols

1. Introduction

Diabetes mellitus, by virtue of its frequency and severity of its metabolic effects on the mother and the fetus, has long been one of the most common and significant medical complications during pregnancy. When pregnancy occurred in women with diabetes mellitus, both maternal and fetal mortalities and morbidities were extremely high. It has become increasingly clear that normal metabolic fuel is essential for normal embryogenesis, and that normalization of the maternal carbohydrate metabolism is best accomplished prior to conception rather than after organogenesis. As pregnancy proceeds, the prevention of maternal complications and perinatal morbidity and mortality requires continued normalization of carbohydrate metabolism and close surveillance of the fetal condition [1]. Improved techniques and further modification of present devices, and possibly the development of new ones, will enhance methods of glucose evaluation and control. New devices and techniques would be expected to lead to an understanding of the mechanism of pregnancy complicated by abnormal glucose metabolism, and be directed toward advancing the treatment of diabetes mellitus during pregnancy closer to the levels of maternal and fetal morbidities and mortalities seen in the general population. Gas chromatographic techniques [2–7] and gas chromatograhy–mass spectrometric methods [8–10] have been used for multi-component analysis of sugars and polyols in cerebrospinal fluid, plasma and urine. However, purification procedures prior to analyses are relatively time consuming and a limited number of samples is processed simultaneously. In 1991,

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Shoemaker et al. [11] first reported a procedure using urease for urine sample preparation. Matsumoto et al. [12] refined and simplified that procedure, which allows us to analyse urinary sugar and sugar alcohols in a large number of samples rapidly using GC–MS. Since little is known about urinary sugar and sugar alcohols concentrations in normal pregnancy, our attempts have been focussed on the application of this method to an understanding of the metabolic disarrangement of carbohydrates during pregnancy, and, further, to evaluate the abnormal carbohydrate metabolism during pregnancy.

2. Experimental

2.1. Chemicals

Solvents and reagents were of the highest purity commercially available, and were obtained from the following sources: methanol from Wako Junyaku (Osaka, Japan); *N*,*O*-bis(trimethylsilyl)trifluoroacetamide from Wako Junyaku (Osaka, Japan); trimethylchlorosilane from Tokyo Kasei (Tokyo, Japan); *n*-heptadecanoic acid from Tokyo Kasei (Tokyo, Japan); urease type C-3 from Sigma (St. Louis, USA).

2.2. Subjects

From a total of 536 samples, 292 were obtained in the first trimester of pregnancy; 159 in the second trimester; and 85 in the third trimester. Subjects included 353 women with normal pregnancies, and 48 women with suspected abnormal carbohydrate metabolism as defined by urinary glucose values. When the urinary glucose value in each trimester was less than two standard deviations above the mean, a pregnancy was tentatively called normal. Forty-one samples from 41 non-pregnant women served as controls. The control subjects were matched with the pregnant women by age. Urine specimens were collected 2 h after a meal. In order to evaluate the daily profile of urinary sugar and sugar alcohols, and to evaluate fluctuations of these compounds during a 75 g oral glucose tolerance test, urine samples were collected just before and 2 h after each meal from seven healthy non-pregnant women and one pregnant woman, respectively.

2.3. Sample preparation

Urine samples were prepared according to the method with modifications of Shoemaker et al. [11]. A hundred μl of urine was incubated with 30 units of urease at 37°C for 10 min. After adding an internal standard (20 μg of *n*-heptadecanoic acid), the sample was centrifuged to deproteinize by adding 900 μl of ethanol, then the supernatant was evaporated. The residue was completely dried under a nitrogen stream for 5 min and derivatized with 100 μl of *N*,*O*-bis (trimethylsilyl) trifluoroacetamide and 10 μl of trimethylchlorosilane at 80°C for 30 min.

2.4. Gas chromatography–mass spectrometry

A QP5050 gas chromatograph–mass spectrometer computer system (Shimadzu, Kyoto, Japan) was used with an Ultra Alloy capillary column (30 m×0.25 mm I.D. with 0.25 μm film thickness, Frontier Lab, Fukushima, Japan). The temperature was programmed to increase from 60°C to 325°C at 17°C min^{-1}, then to remain constant at 325°C for 3 min. One μl of derivatized sample was injected at a split ratio of 20:1. The time required for one sample analysis was 20 min. The mass chromatographic quantitation of urinary sugar and sugar alcohols was based on the relative intensity of the ion peaks compared with the corresponding ion of *n*-heptadecanoic acid. A fragment ion of each sugar and sugar alcohol [glucose: *m*/*z* 435 (M-90); sorbitol: *m*/*z* 421 (M-193); fructose: *m*/*z* 437 (M-103); myo-inositol: *m*/*z* 507 (M-105); 1,5-AG: *m*/*z* 259 (M-193)] was chosen for the quantifications (Fig. 1). Standard curves were drawn by plotting the ratio between the index compound and the internal standard against the known amount of the index compound. Glucose, galactose, and fructose are separated as two peaks (α, β-anomer) on the chromatogram. Because the ratio of the two peaks for glucose reveals a constant, the β-anomer was quantified. The ratio of the two peaks for fructose fluctuated, so that the value for fructose obtained was the sum of two peaks. Concentrations of the compounds were corrected for the concentration of creatinine determined by Jaffe's method

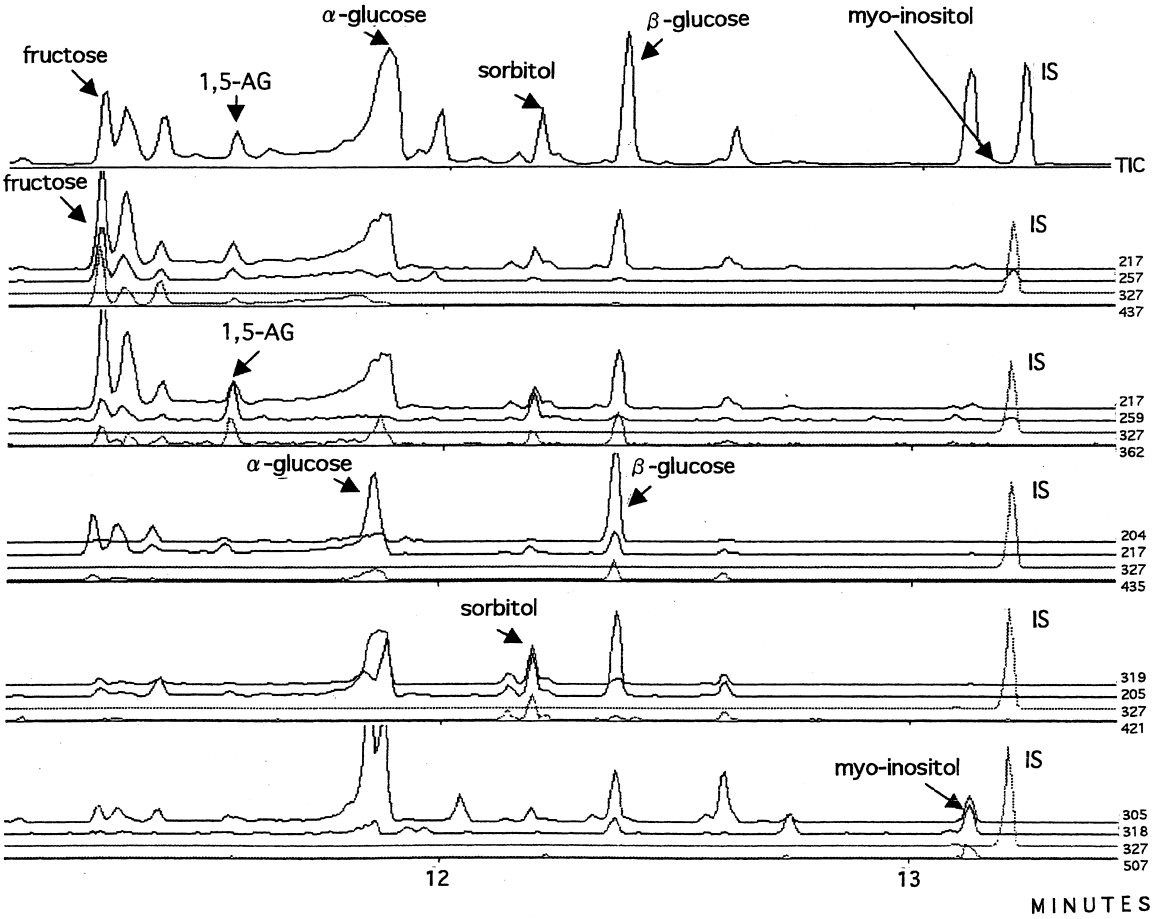

Fig. 1. Tic chromatogram and mass chromatograms of TMS derivatives of urinary sugar and sugar alcohols. IS denotes *n*-heptadecanoic acid as an internal standard. *x*-axis indicates minutes of retention time.

[13]. The precision of the method was checked by pooled specimen accompanying the subjects' samples. Statistical significance was determined by Student's *t*-test for paired and unpaired data.

3. Results

3.1. Evaluation of the mass spectral analysis

The Tic chromatogram and mass chromatograms of the TMS derivatives of urinary sugar and sugar alcohols from a pregnant subject are depicted in Fig. 1. The standard curves were linear over the ranges 5.0–200.0 μg/100 μl ($r=0.9999$) for glucose, 2.5–20.0 μg/100 μl ($r=0.9991$) for sorbitol, 5.0–80.0 μg/100 μl ($r=0.9953$) for fructose, 2.5–20.0 μg/100 μl ($r=0.9989$) for myo-inositol, and 1.0–8.0 μg/100 μl ($r=0.9992$) for 1,5-AG, respectively. Five aliquots of the same urine samples were prepared and seven injections in each aliquot were performed during an interval of 12 h to determine the intra-assay variation. There were no significant changes in the levels of urinary sugar and sugar alcohols (Table 1). To determine the interassay variation, aliquots of the pooled urine sample were analyzed 30 times over 40 days. The CVs were 7.7% (856.1 ± 65.6 μg ml^{-1}, mean±SD $n=30$) for glucose, 5.5% (5.6 ± 0.3 μg ml^{-1}) for sorbitol, 8.6% (5.6 ± 0.5 μg ml^{-1}) for fructose, 14.2% (6.4 ± 0.9

Table 1
Intra-assay variability, each derivatized sample from the same urine specimen was analyzed seven times[a]

Sample no.	Glucose		Sorbitol		Fructose		Myo-inositol		1,5-AG	
	$M\pm$SD ($n=7$)	CV	$M\pm$SD ($n=7$)	CV	$M\pm$SD ($n=7$)	CV	$M\pm$SD ($n=7$)	CV	$M\pm$SD ($n=7$)	CV
1	784.9±16.8	2.1	88.3±3.3	3.8	3.4±0.3	8.4	6.3±0.5	7.5	2.6±0.2	7.8
2	779.7±22.9	2.9	92.7±4.0	4.4	3.5±0.2	6.7	6.2±0.1	2.4	2.4±0.2	7.7
3	779.3±20.9	2.7	93.3±1.7	1.8	3.5±0.1	4.2	6.4±0.3	4.5	2.7±0.2	7.5
4	783.0±15.9	2.0	91.7±3.9	4.3	3.4±0.2	8.0	6.6±0.3	4.8	2.5±0.2	7.1
5	757.3±5.5	0.7	85.6±2.1	2.4	3.4±0.2	6.4	5.6±0.4	6.2	2.5±0.2	8.2

[a] Results are expressed as mean±SD $\mu g\,ml^{-1}$ with number of analyses in parentheses. Concentrations are not corrected for the concentration of creatinine. CV denotes coefficient of variation (%).

$\mu g\,ml^{-1}$) for myo-inositol and 11.7% (2.3±0.3 $\mu g\,ml^{-1}$) for 1,5-AG, respectively. The values were not corrected for the concentration of creatinine.

3.2. Daily variation of urinary sugar and sugar alcohols

In order to determine the short-term fluctuations of urinary sugar and sugar alcohols, urine specimens obtained from seven non-pregnant women just before and 2 h after each meal were analyzed. The fluctuation of glucose, sorbitol, myo-inositol, and 1,5-AG were insignificant. The concentration of fructose 2 h after lunch had a higher statistical significance ($p<0.05$) when compared with fructose concentrations at other time points (Fig. 2).

3.3. Concentrations of urinary sugar and sugar alcohols during a 75 g oral glucose tolerance test

Urine samples obtained from a woman during a 75 g oral glucose tolerance test performed at 27 weeks of gestation and the 30th day postpartum were analysed to evaluate the changes of urinary sugar and sugar alcohols levels after oral glucose loading. The profiles of plasma glucose at each time point during pregnancy and the puerperium were classified as exhibiting impaired glucose tolerance, according to the criteria established by the World Health Organization expert committee, and normal glucose tolerance, respectively. The changes in concentrations of urinary sugar and sugar alcohols were significant during pregnancy when compared with those from the puerperal period. The maximum increasing rate of urinary glucose, sorbitol, fructose, myo-inositol and 1,5-AG during pregnancy was 1350%, 100%, 450%, 650% and 200%, respectively, while the increasing rate of serum glucose was 200%. In the puerperium, the responses of urinary sugar and sugar alcohols to glucose loading were not striking.

3.4. Concentrations of sugar and sugar alcohols in women over the course of a normal pregnancy

The urinary sugar and sugar alcohols values obtained from non-pregnant women and from pregnant women at each trimester are illustrated in Fig. 3. The concentrations of sugar and sugar alcohols in normal non-pregnant women and in women with normal pregnancies in the first, second and third trimesters of pregnancy were found to be 67.2±18.8 μg/mg creatinine (mean±SD) ($n=41$), 54.5±27.6 μg/mg creatinine ($n=278$), 52.1±23.2 μg/mg creatinine ($n=139$), 52.1±21.7 μg/mg creatinine ($n=65$) for glucose, respectively; 20.5±16.3 μg/mg creatinine, 57.4±43.4 μg/mg creatinine, 85.8±60.2 μg/mg creatinine, and 99.5±67.2 μg/mg creatinine for sorbitol; 10.5±6.1 μg/mg creatinine, 112.8±115.1 μg/mg creatinine, 92.8±96.9 μg/mg creatinine and 99.5±91.0 μg/mg creatinine for fructose; 16.6±11.3 μg/mg creatinine, 32.4±23.8 μg/mg creatinine, 26.3±20.1 μg/mg creatinine, and 25.1±18.9 μg/mg creatinine for myo-inositol; 4.0±2.3 μg/mg creatinine, 8.1±7.1 μg/mg creatinine, 4.3±4.9 μg/mg creatinine, and 4.1±3.5 μg/mg creatinine for 1,5-AG. The urinary glucose level had a declining statistical significance in the

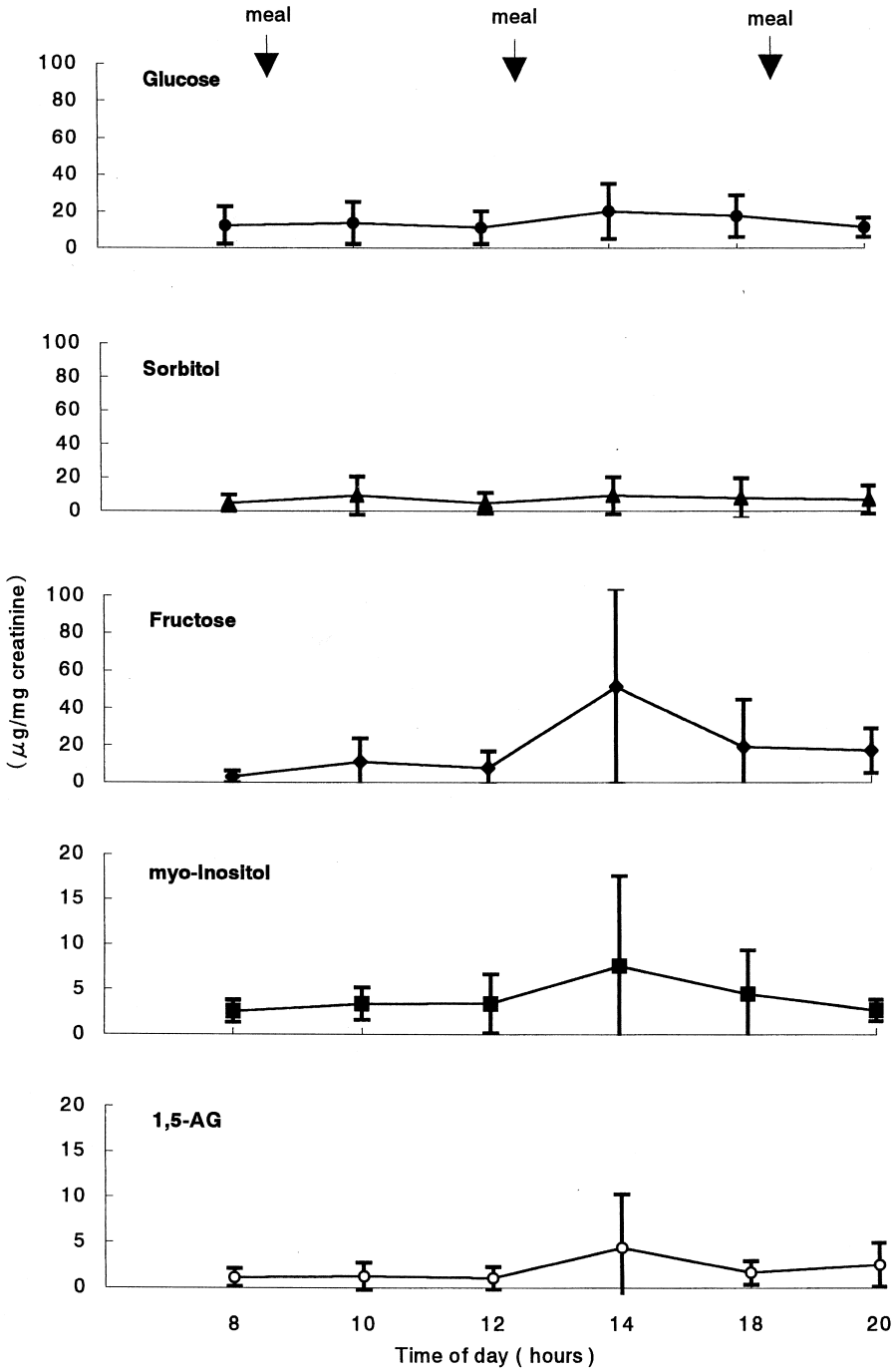

Fig. 2. Daily profiles of urinary sugar and sugar alcohols in healthy non-pregnant women. Urine specimens were collected just before and 2 h after each meal. Bars denote mean±SD for seven subjects.

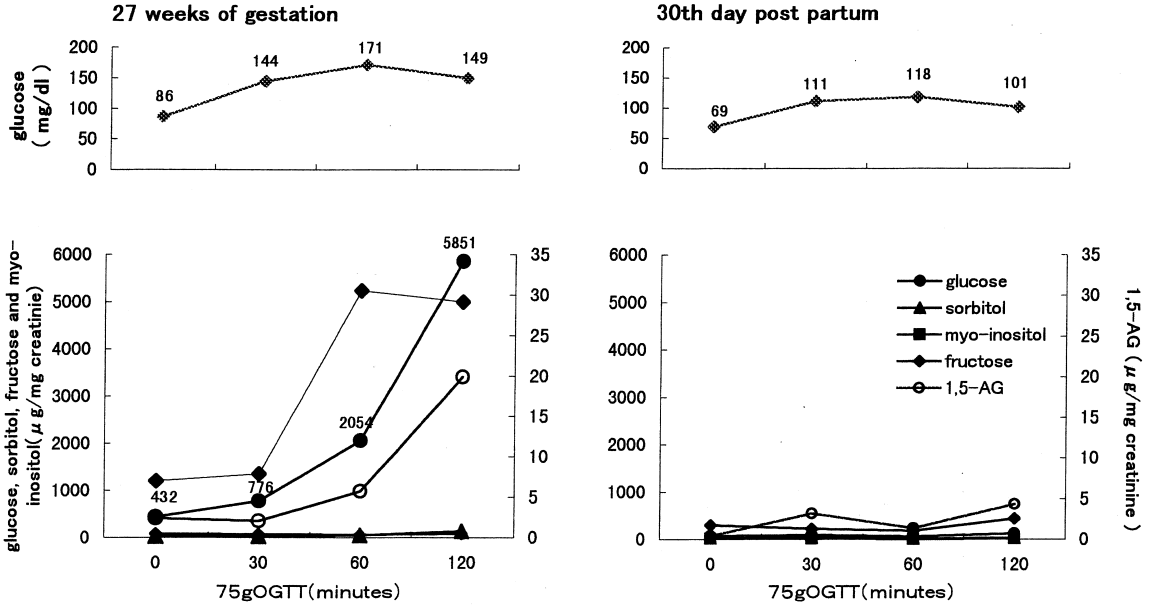

Fig. 3. Plasma values of glucose and urinary sugar and sugar alcohols in subject at 27 weeks of gestation (left) and at the 30th day postpartum (right) during 75 g oral glucose tolerance tests. The upper part of the figure shows changes of plasma glucose; the lower part, urinary sugar and sugar alcohols.

first trimester of pregnancy, and was unchanged in the second and third trimesters.

Sorbitol level increased in the first trimester and steadily increased as the pregnancy progressed. Fructose and myo-inositol levels increased in the first trimester and remained unchanged in the second and third trimesters. 1,5-AG level increased transiently in the first trimester and then declined. The concentration of urinary glucose in non-pregnant women is significantly higher when compared to that in pregnant women ($p<0.01$), while concentrations of sugar alcohols are significantly higher in pregnant women ($p<0.001$ for sorbitol, fructose, myo-inositol and 1,5-AG) (Fig. 4).

3.5. Concentrations of sugar and sugar alcohols in subjects with abnormal glucose metabolism

The urinary concentrations of sugar and sugar alcohols in the first, second and third trimesters of pregnancy in women with abnormal glucose metabolism were 567.7±405.7 μg/mg creatinine ($n=14$), 612.7±529.8 μg/mg creatinine (mean±SD) ($n=20$), and 1242.5±1288.9 μg/mg creatinine ($n=20$) for glucose, respectively; 93.3±38.4 μg/mg creatinine, 235.9±153.9 μg/mg creatinine and 230.0±169.5 μg/mg creatinine for sorbitol; 321.3±260.8 μg/mg creatinine, 184.7±166.7 μg/mg creatinine, and 690.4±741.5 μg/mg creatinine for fructose; 119.9±41.3 μg/mg creatinine, 124.1±58.7 μg/mg creatinine, and 144.5±107.8 μg/mg creatinine for myo-inositol; 52.4±39.8 μg/mg creatinine, 16.2±10.9 μg/mg creatinine, and 30.7±25.1 μg/mg creatinine for 1,5-AG. The level of each sugar and sugar alcohol increased and the standard deviation became wider with advancing gestation (Fig. 5).

4. Discussion

Since a defined and simplified method for the general analysis of urinary metabolites after urease treatment was developed [12], attempts have been made to apply the comprehensive method to the analysis of urinary organic acids [14–16], amino acids, sugars and sugar alcohols [17,18], sugar acids, and nucleic acid bases. The method is proven to be

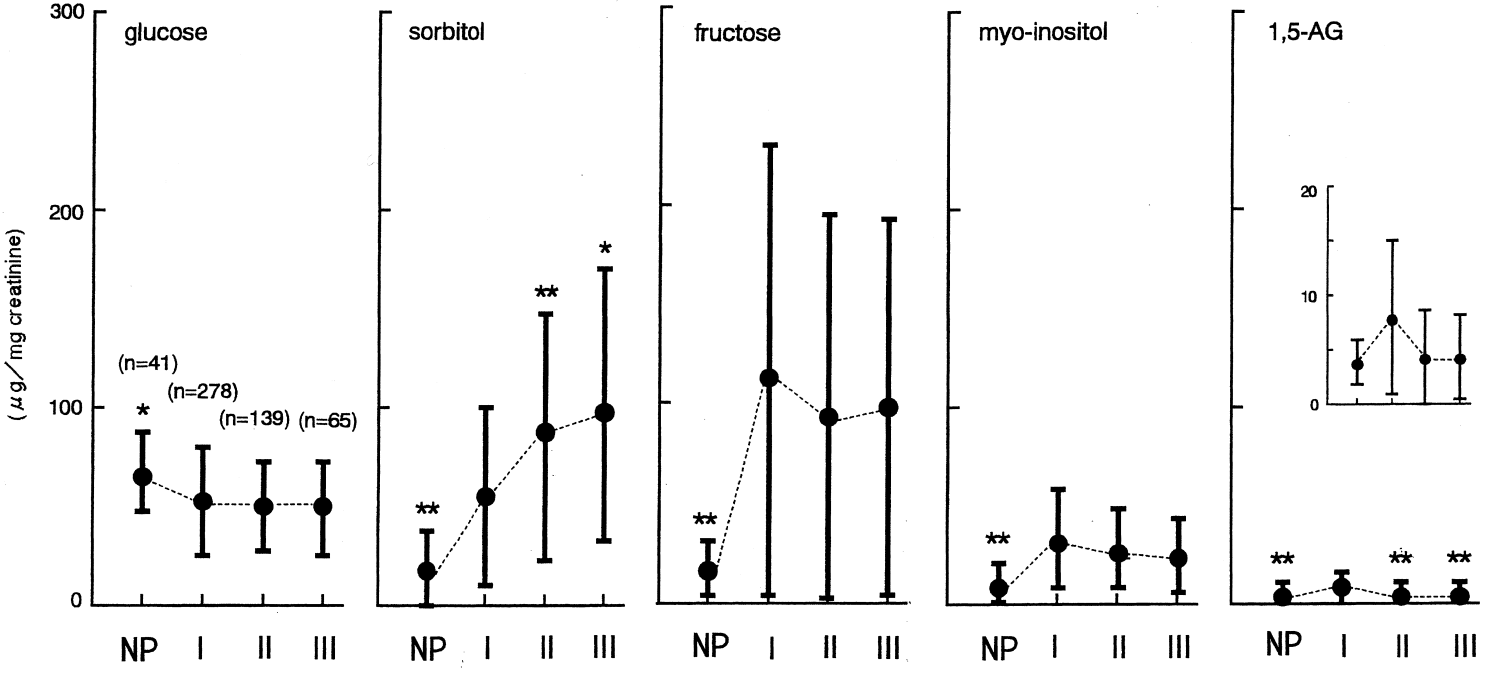

Fig. 4. Concentrations of urinary sugar and sugar alcohols in subjects during normal pregnancy. Bars denote mean±SD for each trimester of pregnancy. NP, I, II, and III represent non-pregnant state serving as controls, the first, second, and third trimester of pregnancy, respectively; *, $p<0.01$,**, $p<0.001$ vs. the first trimester of pregnancy.

rapid and highly reproducible. The time required for sample preparation and GC–MS analysis is 3 h for a batch of 30 samples, and 20 min for one sample, respectively. Inter- and intra-assay variations were observed to be sufficiently small for clinical study. In the field of obstetrics, pregnancy complicated by abnormal carbohydrate metabolism has been one of the most significant problems because of its frequency and the severity of its metabolic effects. It has become clear that the subclinical maternal abnormal carbohydrate metabolism including gestational diabetes mellitus and impaired glucose tolerance, is associated with neonatal morbidities and increased risk of later maternal glucose intolerance in the non-pregnant state.

Since diabetogenic changes occur during normal pregnancy and the exaggerated effects are thought to contribute to the development of maternal glucose intolerance, our interest has been focussed on understanding the pathophysiology of carbohydrate metabolism during pregnancy. To our knowledge, little information exists regarding the changes in urinary levels of sugar and sugar alcohols during normal pregnancy, which permits us to evaluate the metabolic disarrangement of carbohydrates from the viewpoint of glucose excursion and glucose fluxes. In this simultaneous analysis of urinary sugar and sugar alcohols, glucose, sorbitol, fructose, myo-inositol and 1,5-AG were chosen based on the experimental evidence that, as a result of hyperglycemia, the excess free glucose in tissues leads to an increased flux of glucose through the polyol pathway, including sorbitol and fructose, and a depletion of cellular myo-inositol [19–21], and that resorption of 1,5-AG by renal tubules is known to compete with that of glucose in the presence of hyperglycemia [22]. The serum concentration of 1,5-AG is reduced in patients with diabetes mellitus [23,24] and in normally pregnant women [25], which provides a sensitive indicator of glycemic control.

The diurnal variations of urinary sugar and sugar alcohols in healthy non-pregnant subjects indicate that the postprandial levels of urinary sugar and sugar alcohols might be accentuated by diet. Parallel changes were observed in the concentrations of 1,5-AG, fructose and glucose in the urine collected at every time point during a 75 g oral glucose tolerance test. The result supports the evidence that 1,5-AG and fructose are reabsorbed in the renal tubules by common transport system [22]. The changes of these compounds during a 75 g oral glucose tolerance test also reveals that the dynamic ranges for urinary sugar and sugar alcohol levels, particularly in pregnant subjects, are wider than those found for blood glucose. Against this background, it is suggested that a 2-h postprandial specimen is more suitable for detecting a mild disruption of carbohydrate metabolism than that collected in the fasting state.

The concentrations of urinary glucose, fructose and myo-inositol remained unchanged during pregnancy, while that of sorbitol increased with advancing gestational age. The concentration of 1,5-AG increased in the first trimester of pregnancy and then declined. The concentration of urinary glucose in non-pregnant women is significantly higher when compared to that in pregnant women, while concentrations of sugar alcohols during pregnancy are significantly higher in pregnant women. The higher levels of urinary sugar alcohols might reflect either an activation of the sorbitol pathway as has been observed under hyperglycemia [19–21] and/or mild metabolic disarrangement of carbohydrates that go undetected by all other diabetic indicators. The lower level of urinary glucose in pregnant subjects is controversial. It might be attributed to increased consumption of glucose in the fetal compartment which results in a decline of renal filtration of glucose. To prove any of these assumptions, an animal study with stable isotopes would be required. The lower levels of glucose in the pregnant subjects might also derive from sample manipulation. In this pilot study, upper limits for normal were tentatively defined as two standard deviations above the mean for urinary glucose concentrations in each trimester of pregnancy, which corresponded to 90% of the entire samples analyzed. A 10% portion of the samples was excluded as abnormal, a manipulation which appears to have been valid because the percentage is consistent with the positive rate in the screening test for abnormal carbohydrate metabolism performed at 24–28 weeks of gestation using a 50 g glucose challenge test. In the non-selected population, the concentrations of urinary glucose during pregnancy were higher when compared with those of non-pregnant subjects; 67.2 ± 18.8 μg/mg creatinine (mean ± SD) ($n = 41$) in the non-pregnant state,

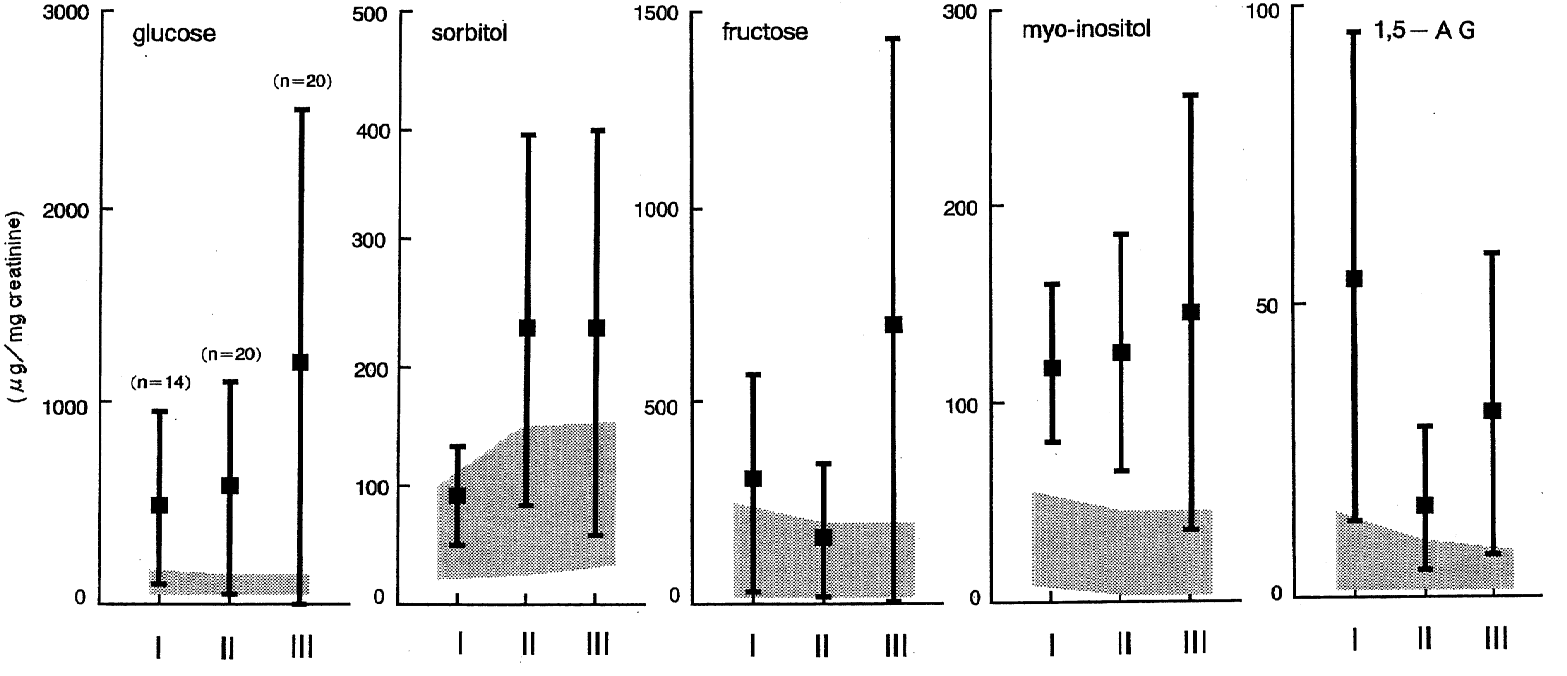

Fig. 5. Concentrations of urinary sugar and sugar alcohols of women with suspected abnormal carbohydrate metabolism, tentatively defined by urinary glucose values. Bars denote mean±SD for each trimester of pregnancy. Shaded areas denote mean±SD for each trimester of normal pregnancy. I, II, and III represent the first, second, and third trimester of pregnancy, respectively.

79.1±174.5 μg/mg creatinine ($n=292$) in the first trimester of pregnancy, 122.6±328.7 μg/mg creatinine ($n=159$) in the second trimester, and 292.8±955.0 μg/mg creatinine ($n=101$) in the third trimester. Regardless of the sample manipulation, the levels of sugar alcohols when compared with that of glucose were demonstrated to be high in pregnant subjects in selected and non-selected populations. The mean values and the standard deviations in the subjects with tentatively defined abnormal carbohydrate metabolism were significantly high and wide, respectively, when compared with those observed in normally pregnant subjects. Because normal values of urinary sugar and sugar alcohols have not yet been elucidated, metabolically normal subjects might be included in a portion of the samples. The wider ranges of standard deviations, together with responses of sugar and sugar alcohols to oral glucose load again imply that the normal upper limit for each compound is presumed to be higher than expected and these compounds might be sensitive alternatives for detecting a mild disruption of carbohydrate metabolism that cannot be detected by conventional methods for glucose monitoring.

This pilot study is part of a program aimed at the development of generally applicable methods for the evaluation of carbohydrate metabolism during pregnancy and for earlier detection and management of abnormal carbohydrate metabolism in pregnant women. Prior to clinical application, the upper limits for normal urinary sugar and sugar alcohols concentrations in pregnant subjects should be determined. In order to establish norms, a study is required on the basis of the statistical model, and there is a need to examine the correlation between the statistical values and the data, per subject, relating to the maternal carbohydrate metabolism determined by oral glucose tolerance testing and perinatal fetal morbidities.

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