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Simultaneous measurement of urinary polyols using gas chromatography/mass spectrometry

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Abstract

In the present study, we simultaneously measured several polyols, such as adonitol, arabitol, dulcitol, glucose, myo-inositol, mannitol, sorbitol, and xylitol, in urine by gas chromatography/mass spectrometry–positive chemical ionization. We also examined possible relationship between the levels of these metabolites and age in normal individuals. In order to proceed to its quantification by GC/MS, 200 μL of a urine sample were diluted with 3 mL of distilled water, lyophilized, acetylated, and then analyzed them. Using this method, we were able to quantify as little as 0.5–1.0 ng/μL, and we made the calibration curves to be linear from 0.25 to 250 ng/μL ($r^2 > 0.991$). Analytical recoveries were over 89.4%, and the inter-day and intra-day variability for accuracy and reproducibility was less than 20%. In the normal urine sample, the levels of polyols were gender-differentiated and age-related. This simple GC/MS method is sensitive and allows the measurement of wide ranges of polyols using small amounts of urine. We conclude that the quantitation of urinary polyols using GC/MS appears to be a clinically useful method for assessing polyol-pathway activity.
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Keywords: Polyol-pathway; Urine; Gas chromatography/mass spectrometry–positive chemical ionization; Sorbitol; Glucose

1. Introduction

Polyol, hydrogenated carbohydrate, has low digestibility, is slowly absorbed in the small intestine, excreted via kidneys, oxidized directly or converted by glycogen or glucose in the liver, and is almost completely fermented in the large intestine. Because of its low carcinogenicity, low glycemia, low insulinemia, and low energy value, polyols are healthful food ingredients and beneficial for good dental health [1,2]. However, in the aldose-reductase deficiencies under hyperglycemia conditions, polyol pathways accelerate the accumulation of polyols which leads to early tissue damage and new inborn error [3–7]. Since polyols are potentially toxic to the central nervous system, deleterious long-term effects of delayed motor development, mood disorders, and facial dysmorphysm could result [8,9]. Therefore, screening of polyols in biological fluids is of interest for nutrition and clinical diagnoses. Many research papers describe the detecting of polyols using various methods. An automated enzymatic assay has been used to study carbohydrates in body fluids [10]. Researchers have also developed many high-performance liquid chromatographic (HPLC) methods with electrochemical and light-scattering detection using anion-exchange separation without pre- or post-column derivatizations. In addition, researchers have developed a method of pulsed ampherometric detection to monitor carbohydrates, including polyols, in biological fluid [11–13]. Recently, some have used the spectroscopic method for clinical purposes in vivo and in vitro NMR spectroscopy [14]. This NMR study could not calculate the concentration of polyols in urine or biological fluid because of overlapping with background resonances. So, various gas chromatography/mass spectrometry (GC/MS) methods have been used to quantify polyols in biological fluids [15–17]. Most of these methods are time-consuming and require digestion procedures. In the present study, we describe a method for measuring of urinary polyols using GC/MS without solvent extraction or column-sample clean-up. The method depends on the use of simple a syringe-filter and one-step acetylation. We quantified the level of urinary polyols in normal

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men and women and examined the effect of polyol-pathway according to several age levels. Our study appears to be a clinically useful method for assessing polyol-pathway activity.

2. Experimental

2.1. Materials and chemicals

An anhydrous acetic anhydride and pyridine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethyl acetate was of HPLC grade from J.T. Baker (Phillipsburg, NJ, USA). The standard polyols, arabitol, adonitol, xylitol, glucose, mannitol, dulcitol, sorbitol, and myo-inositol, were purchased from Sigma–Aldrich. A deuterated glucose (6,6-$^2H_2$-glucose; 6,6-D_2-G) as an internal standard (IS) was obtained from Cambridge Isotope Lab. (Andover MA, USA). A stock solution of polyols was prepared at concentration of 1 mg/mL in de-ionized water and stored at −20 °C until use. The solution was diluted further with de-ionized water to desired concentrations (1–100 μg/mL) before use. The 6,6-D_2-G was prepared at concentration of 100 μg/mL in the de-ionized water.

2.2. Urine collection

Urine samples were collected from 50 healthy male subjects aged 30–82 years (mean ± S.D.; 46.0 ± 10.0) and 52 healthy female subjects aged 29–71 years (mean ± S.D.; 42.5 ± 8.8) in Kyunghee Medical Center, Kyunghee University, Seoul, Korea. All urine samples were stored at −20 °C until analyzed. The urinary creatinine value was measured by a Jaffé method [18].

2.3. Sample preparation of polyols in the urine

Following fortification of relevant human urine samples with the 6,6-D_2-G as an IS at a level of 125 pg/μL, 200 μL aliquots of the urine sample were diluted with 3 mL of de-ionized water. The diluted urine samples were mixed well and filtered through a 0.22-μM cellulose-acetate syringe filter (Cameo syringe filter, GE Osmonics Labstore, Minnetonka, MN, USA). The filtered samples were lyophilized overnight. The dried residues were added to 300 μL of an acetic anhydride-pyridine (2:1, v/v) mixture reagent and heated it at 60 °C for 30 min. After being cooled, the acetylated samples were blown to dryness under nitrogen gas and re-dissolved in 500 μL of ethyl acetate. We injected approximately 2 μL of derivatized aliquots onto the GC/MS.

2.4. Gas chromatography/mass spectrometry

All experiments were performed on a ThermoFinnigan Trace gas chromatography interfaced to a ThermoFinnigan PolarisQ ion trap mass spectrometer (ThermoFinnigan, Austin, Texas, USA) with positive chemical ionization mode at a 1.2 mL of methane flow. The analytical column was an SPB-1701 column (15 m × 0.25 mm i.d., 0.25 μm film thickness; J&W, Folsom, CA, USA) and the carrier gas was helium at a column flow of 1.2 mL/min at 80 °C. The splitless injection mode was used with 1 min purge off time. The temperature program follows: initial temperature: 60 °C; held for 1 min, first program rate: 60 °C/min to 180 °C, held for 1 min, second program rate: 5 °C/min to 220 °C, held for 1 min, and final program rate: 50 °C/min to 250 °C, held for 1 min. In the SIM mode, we achieved the peak identification by comparing the retention times and matching the area ratios of these polyols base ion to IS. The most intense ions were at *m*/*z* 303 for adonitol, arabitol, and xylitol, at *m*/*z* 169 for glucose, at *m*/*z* 171 for 6,6-D_2-G as an IS, at *m*/*z* 373 for mannitol, dulcitol, and sorbitol, and at *m*/*z* 375 for myo-inositol with 50 ms of dwell time.

2.5. Method validation

Intra- and inter-day assays were fortified with 0.25, 1.0, and 10 μg of polyols into 200 μL of de-ionized water. The calibration samples were fortified with increasing amounts ranging from 0.25 to 250 ng/μL for each polyol standards in the same manner as described in the sample-preparation section. The detection limit for each polyols was calculated based on the weight, giving a signal three times the peak-to-peak noise of the background signal. In order to obtain the linearity of SIM responses and to plot calibration curves for the quantitative measurement of polyols, a least-squares regression analysis was performed with increasing area ratios of polyol to the IS.

3. Results and discussion

3.1. Gas chromatography/mass spectrometric analysis

In order to detect acetylated polyols, we used GC/MS–SIM with methane in a positive chemical ionization mode set to monitoring at *m*/*z* 303, 169, 171, 373, and 375. Each resolved polyols displayed a single peak with symmetric shape (Fig. 1; up layer), except overlapping for the pairs of glucose and 6,6-D_2-G, and sorbitol and myo-inositol. The chromatographically unresolved compounds were sufficiently distinguishable to be quantified in the SIM mode using different selected *m*/*z* values for each, that were the pairs of glucose and 6,6-D_2-G at *m*/*z* 169 and 171, and sorbitol and myo-inositol at *m*/*z* 375 and *m*/*z* 373. Fig. 1 also depicts the mass spectra of the acetylated polyol that yielded the characteristic mass spectrum of $[M\text{-}OCOCH_3]^+$ ion as a base peak, except glucose at *m*/*z* 169 and 6,6-D_2-G at *m*/*z* 171.

3.2. Range of linearity and limit-of-detection

Table 1 depicts the GC/MS data of polyols as an acetylated derivative. The linear responses to polyols were obtained in the range of 0.25–250 ng/μL with a correlation coefficient range of 0.991–1.000 and the limit-of-detection ranged from 0.5 to 1.0 ng/μL. The intra- and inter-day assay variances for the three concentrations are displayed in Table 2. The coefficient of variation (CV, %) were 1.2–37.3%, and the range of analytical recoveries was 89.4–114.4% (Table 2).

3.3. Urine analysis

We simply measured polyols in urine samples from healthy men (age 30–82; mean ± S.D.: 46.0 ± 10.0; $n = 50$) and women

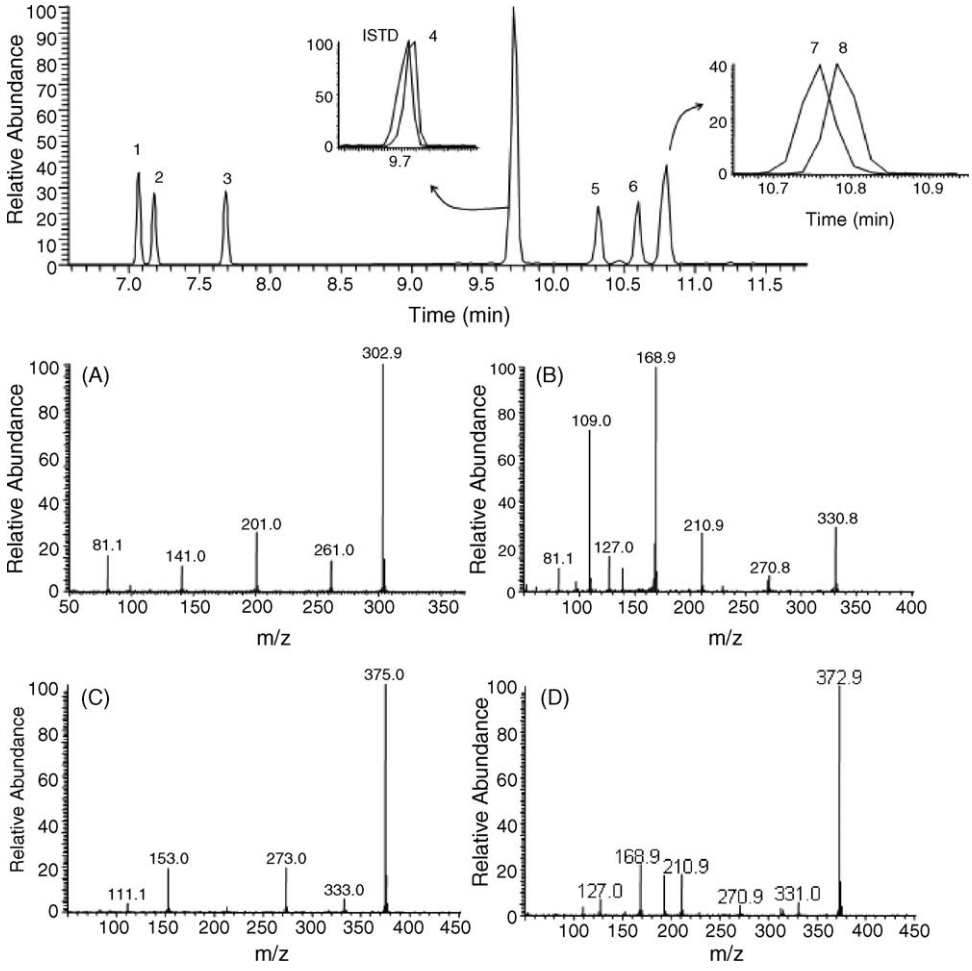

Fig. 1. Representative total ion chromatogram and mass spectra of standard polyols as acetylated derivative obtained in SIM mode separated on an SPB-1701 (15 m × 0.25 mm i.d., 0.25 μm film thickness) column. The magnified figures were ion chromatogram at *m/z* 169 and 171 for peak 4 and ISTD, at *m/z* 375 for peak 7, and at *m/z* 373 for peak 8. Peak 1: adonitol at 7.08; peak 2: arabitol at 7.18; peak 3: xylitol at 7.69; peak 4: glucose at 9.73; peak 5: mannitol at 10.32; peak 6: dulcitol at 0.60; peak 7: sorbitol at 10.78; peak 8: myo-inositol at 10.82; ISTD: 6,6-D2 glucose at 9.72 min. Mass spectrum: (A) adonitol, arabitol and xylitol; (B) glucose; (C) mannitol, dulcitol and sorbitol; (D) myo-inositol.

(age 29–71; mean ± S.D.: 42.5 ± 8.8; $n = 52$). Fig. 2 shows typical gas chromatograms of urinary polyols in normal man and woman. The pattern of polyols was that arabitol and mannitol were major components, and the others were less than 20% relatively to arabitol. Table 3 shows the distribution of urinary polyol levels in normal men and women within a 10-year age group. The detected amounts (ng/μL) from the described method were converted to μmol/L and then divided by g/L creatinine

Table 1
GC/MS data of polyols as acetylated derivative

Polyols	Mw	RRT[a]	Quantitative ion (*m/z*)	Detection limit (ng/μL)	Calibration curve $Y = aX + b$		Linearity[b] (r^2)
					a	*b*	
Adonitol	362	0.72	303	1.0	0.2261	−0.4800	1.000
Arabitol	362	0.73	303	1.0	0.1899	−0.3546	1.000
Xylitol	362	0.79	303	1.0	0.2983	−1.2010	0.996
Glucose	390	1.00	169	1.0	0.0429	−0.2555	0.991
6,6-D_2 glucose	392	1.00	171	–	–	–	–
Mannitol	434	1.07	375	1.0	0.0867	0.0939	1.000
Dulcitol	434	1.10	375	1.0	0.2909	−0.8212	0.999
Sorbitol	434	1.11	375	0.5	0.0985	0.1072	0.999
myo-Inositol	432	1.12	373	0.5	0.2959	−0.6453	0.999

The calibration samples were fortified with increasing amounts ranging from 0.25 to 250 ng/μL for each polyol standards into de-ionized water as the same manner described in the sample-preparation section.

[a] Retention time relative to that of 6,6-D_2 glucose.

[b] Linearity was described with linear correlation coefficients for calibration curves.

Table 2
Validation of the method for the assay of polyols

Polyols	Added amounts	Intra-day ($n = 5$)		Inter-day[a] ($n = 5$)		Mean recovery[b]
		Mean ± S.D.	CV (%)	Mean ± S.D.	CV (%)	Mean ± S.D.
Adonitol	1.3	1.2 ± 0.1	4.0	1.5 ± 0.4	13.0	102.4 ± 9.0
	5.0	5.2 ± 0.2	4.3	4.8 ± 0.4	8.2	
	50.0	51.9 ± 1.5	2.8	47.6 ± 0.6	1.2	
Arabitol	1.3	1.5 ± 0.1	7.6	1.5 ± 0.4	26.4	114.4 ± 8.2
	5.0	5.9 ± 0.3	5.2	4.9 ± 0.4	7.3	
	50.0	57.5 ± 1.5	2.5	59.0 ± 5.1	8.7	
Xylitol	1.3	1.0 ± 0.2	16.4	1.4 ± 0.4	25.8	106.7 ± 17.9
	5.0	6.0 ± 0.2	2.7	6.3 ± 0.3	4.5	
	50.0	53.9 ± 2.7	5.1	45.0 ± 1.9	4.3	
Glucose	1.3	1.4 ± 0.3	18.1	–	–	100.5 ± 13.7
	5.0	4.4 ± 0.2	3.8	6.0 ± 0.5	8.5	
	50.0	48.4 ± 1.5	3.2	44.1 ± 1.5	3.3	
Mannitol	1.3	1.3 ± 0.3	25.0	1.0 ± 0.2	17.9	95.7 ± 14.6
	5.0	3.9 ± 0.3	7.6	4.9 ± 0.8	15.5	
	50.0	55.2 ± 1.6	2.9	52.1 ± 3.1	5.9	
Dulcitol	1.3	1.0 ± 0.4	37.3	1.1 ± 0.4	36.1	106.0 ± 16.3
	5.0	5.8 ± 0.2	3.6	6.0 ± 0.3	5.7	
	50.0	54.3 ± 2.2	4.0	60.0 ± 3.3	5.5	
Sorbitol	1.3	1.0 ± 0.2	17.3	0.9 ± 0.1	6.4	89.4 ± 16.4
	5.0	4.1 ± 0.6	13.5	4.4 ± 1.0	23.6	
	50.0	54.1 ± 2.5	4.6	55.0 ± 4.9	8.9	
myo-Inositol	1.3	1.3 ± 0.3	20.0	1.3 ± 0.3	22.2	103.4 ± 10.2
	5.0	6.0 ± 0.2	3.5	5.1 ± 0.3	4.9	
	50.0	47.5 ± 2.4	5.0	45.5 ± 3.1	6.7	

Unit is ng/μL; validation assays were fortified with 0.25, 1.0, and 10 μg of polyols into 200 μL of de-ionized water as described in the sample preparation section.
[a] Intra-day precision test was performed using the distilled water within the same day, and Inter-day was performed within 5 days.
[b] Recovery was expressed as the mean values for the three concentrations with S.D.; S.D., standard deviation; CV: coefficient of variation.

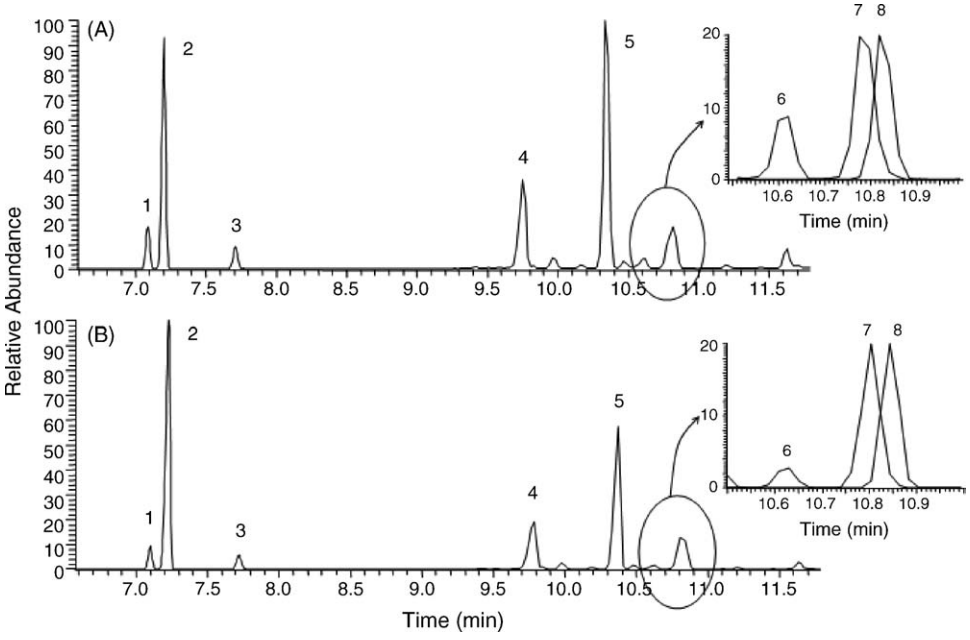

Fig. 2. Urinary profile of polyols as acetylated derivative in man (A) and woman (B) obtained in SIM mode separated on an SPB-1701 (15 m × 0.25 mm i.d., 0.25 μm film thickness) column. The magnified figures were ion chromatogram at *m/z* 375 for peaks 6 and 7 and at *m/z* 373 for peak 8. Peak 1: adonitol at 7.08; peak 2: arabitol at 7.18; peak 3: xylitol at 7.69; peak 4: glucose at 9.73; peak 5: mannitol at 10.32; peak 6: dulcitol at 0.60; peak 7: sorbitol at 10.78; peak 8: myo-inositol at 10.82; ISTD: 6,6-D2 glucose at 9.72 min.

Table 3
Distribution of urinary polyol levels in normal men and women within 10-year age group

	Total	Range	30–39 year	40–49 year	50–59 year	<60 year
	46.0 ± 10.0 (n = 50)	30–82	36.2 ± 2.7 (n = 15)	44.1 ± 3.0 (n = 20)	52.7 ± 2.0 (n = 10)	66.8 ± 8.7 (n = 5)
Men; mean ± S.D.; μmol/g creatinine[a]						
Adonitol	44.3 ± 21.1	17.8–124.8	33.6 ± 15.8	43.3 ± 15.5	55.2 ± 28.0	61.5 ± 20.1
Arabitol	565.7 ± 885.5	183.5–6275.8	312.6 ± 64.9	418.7 ± 191.0	1209.3 ± 1814.2	562.4 ± 260.0
Xylitol	71.8 ± 44.2	26.1–212.1	54.4 ± 30.9	63.1 ± 32.8	103.6 ± 54.2	94.5 ± 60.0
Glucose	383.5 ± 230.5	98.6–1164.5	349.1 ± 237.4	335.5 ± 179.2	470.9 ± 207.1	525.2 ± 360.1
Mannitol	498.4 ± 554.1	45.5–2837.9	256.9 ± 199.3	509.6 ± 472.3	701.6 ± 561.9	783.5 ± 1155.6
Dulcitol	27.7 ± 16.0	12.1–69.6	22.7 ± 14.2	25.1 ± 9.8	35.6 ± 18.3	38.4 ± 26.5
Sorbitol	42.2 ± 39.0	8.4–272.0	27.5 ± 20.5	36.5 ± 15.5	67.3 ± 73.2	59.5 ± 12.2
myo-Inositol	62.4 ± 57.1	3.4–234.3	43.0 ± 26.9	49.3 ± 37.7	87.1 ± 77.4	123.4 ± 83.4
	Total	Range	30–39 year	40–49 year	50–59 year	<60 year
	42.5 ± 6.4 (n = 52)	29–71	35.5 ± 2.8[b] (n = 21)	44.0 ± 3.0 (n = 20)	56.7 ± 2.1 (n = 7)	66.5 ± 6.4 (n = 4)
Women; mean ± S.D.; μmol/g creatinine						
Adonitol	90.3 ± 53.5	29.3–256.7	70.7 ± 37.3	107.3 ± 48.7	116.3 ± 122.6	46.3 ± 19.3
Arabitol	900.5 ± 611.6	246.4–3099.7	699.8 ± 301.8	1024.1 ± 626.9	1561.0 ± 1279.8	340.9 ± 11.1
Xylitol	131.1 ± 77.6	42.9–449.8	105.7 ± 39.9	140.0 ± 67.9	256.1 ± 172.3	61.9 ± 13.5
Glucose	729.2 ± 425.3	308.0–2356.9	617.8 ± 247.9	805.1 ± 457.0	1051.2 ± 881.9	416.9 ± 73.4
Mannitol	1517.3 ± 1518.5	99.5–6284.3	1652.4 ± 2032.3	1522.6 ± 1167.0	1157.7 ± 753.0	924.2 ± 970.0
Dulcitol	48.2 ± 22.4	13.5–121.9	44.1 ± 16.6	51.3 ± 20.5	68.2 ± 49.1	21.5 ± 2.1
Sorbitol	88.3 ± 65.9	13.1–264.8	65.0 ± 49.5	113.1 ± 58.7	100.6 ± 142.5	20.3 ± 10.2
myo-Inositol	132.3 ± 163.7	12.6–929.2	101.8 ± 72.9	130.5 ± 127.6	365.6 ± 488.4	43.7 ± 38.0

The difference in mean values between men and women was not significant; n, number of controls; S.D., standard deviation.
[a] The urinary creatinine value was measured by a Jaffé method.
[b] The age 29 in women merged into 30–39 year group.

amount containing each person that was measured by a Jaffé method. The level of polyols in women was doubly higher than that of the men. The intent of the enzyme expression in the liver, the metabolism and excretion of the polyol, and hormonal state may be different between men and women [19]. Grant et al. have reported that the reductase is sex-specifically regulated in rat. And the activity and protein content of the aldose reductase were greatly affected during the estrous cycle in the

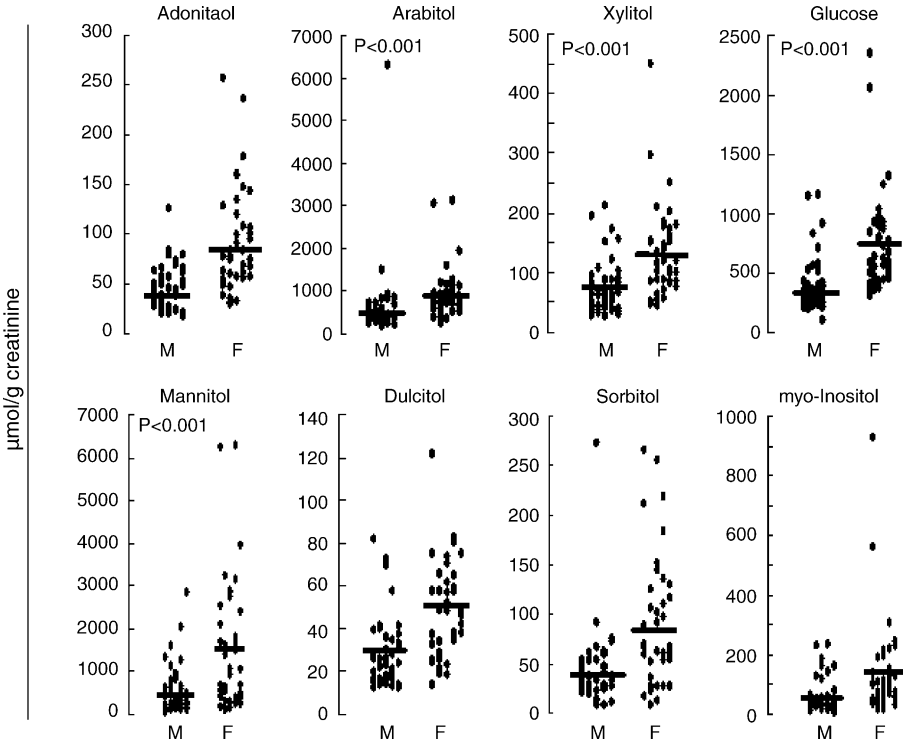

Fig. 3. Comparison of men and women in urinary polyols level. Dulcitol was highly significant in men ($p < 0.001$) and adonitol, sorbitol, and myo-inositol were highly significant in women ($p < 0.001$). M: men; F: women, and dash bar is mean value of each.

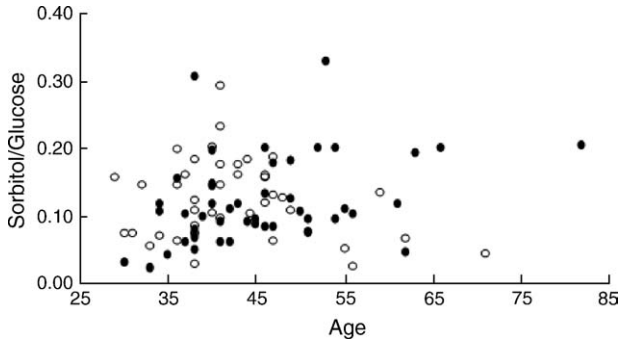

Fig. 4. The ratio of sorbitol to glucose in men and women according to age; $p < 0.001$; dot circle for men; line circle for women.

ovary of rats [20]. So we assumed that sex-hormone was related in the polyol-pathway. The difference in mean values between men and women was not significant. The level of urinary polyols, arabitol, xylitol, glucose, and mannitol, increased with age to become a highly significant factor ($p < 0.001$), while dulcitol was a highly significant in men ($p < 0.001$), and adonitol, sorbitol and myo-inositol were a highly significant in women ($p < 0.001$) (Fig. 3). In the kidneys, the polyol-pathway plays an important role in the excretion of various organic osmolytes. In renal tubular cells, an increment in osmotic pressure causes induction of aldose reductase and reduces polyol dehydrogenase activity. So age-related hyperglycemia may be activated the polyol-pathway both aldose-reductase induction and the intracellular accumulation of sorbitol generated from glucose as a substrate for aldose reductase [8]. In Fig. 4, the ratio of sorbitol to glucose increased with age of the individuals and was highly significant ($p < 0.001$). From this result, we have found that elderly subjects have increased urinary sorbitol levels during age-related hyperglycemia.

4. Conclusion

In the present study, we established simultaneously measurement of several polyols, such as adonitol, arabitol, dulcitol, glucose, myo-inositol, mannitol, sorbitol, and xylitol, in urine using gas chromatography/mass spectrometry–positive chemical ionization, and examined a possible relationship among the levels of polyol, age, and gender in normal, healthy individuals.

The levels of polyols in women were doubly higher than those in the men. This result of gender-differentiation would be affected steroid sex-hormone. The level of urinary polyols increased with age, and the arabitol, xylitol, glucose, mannitol levels were highly significant ($p < 0.001$). Adonitol, sorbitol, and myo-inositol were highly significant for women ($p < 0.001$), and dulcitol was highly significant for men ($p < 0.001$). The ratio of sorbitol to glucose increased with age of the individual with highly significant ($p < 0.001$). Those results may be age-related hyperglycemia. This simple GC/MS method is sensitive and rapid, and it allows for the measurement of wide ranges of polyols using small amounts of urine. The accumulation of polyols has an important role in the peripheral nerves [8,21], diabetic retinopathy [6,22], nephropathy [23], and inborn errors [7,24,25]. For screening of patients for polyol abnormalities, urine is the body fluid of first choice. So the quantitation of urinary polyols using GC/MS appears to be a clinically useful method for assessing polyol-pathway activity.

Acknowledgements

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