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SHORT REPORT

Elevated Serum Sorbitol and not Fructose in Type 2 Diabetic Patients

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Abstract: Reductions in fasting serum fructose or erythrocyte sorbitol have been proposed as markers for early proof of mechanism in clinical development of aldose reductase (AR) inhibitors. However fructose is significantly impacted by meals and evaluation of erythrocyte sorbitol poses technical challenges. To more accurately assess the performance of these markers in biological samples, a gas chromatography-mass spectrometry assay was modified and validated. Serum was collected on three consecutive days from 13 healthy volunteers (HV) and 14 patients with type 2 diabetes mellitus (T2DM), and assayed for sorbitol and fructose using this assay. Serum fructose and sorbitol were relatively constant across the three days. Fasting fructose levels were comparable between the two groups (T2DM: 1.48 ± 0.49 mg/L; HV: 1.39 ± 0.38 mg/L, mean ± standard deviation, $P = 0.61$), but fasting sorbitol levels were significantly higher in diabetics (T2DM: 0.280 ± 0.163 mg/L; HV: 0.164 ± 0.044 mg/L, $P = 0.02$). Feeding resulted in a 5–6 fold increase in serum fructose levels, but only a 5%–10% increase in sorbitol. Only sorbitol remained significantly elevated pre- and post feeding in T2DM patients relative to HV. These data suggest that serum sorbitol may be a robust proof of mechanism biomarker and facilitate dose selection for clinical development of AR inhibitors.

Keywords: diabetic complications, polyol pathway, fructose, sorbitol

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Introduction

According to the National Health and Nutrition Examination Survey (NHANES) 1999–2002, less than 50% of diabetic adults met or exceeded the American Diabetes Association (ADA) recommendation of $HbA_{1c} < 7\%$.[1] These findings were despite the availability of insulin and several drug classes targeting glucose lowering. New classes of drugs targeting microvascular diabetic complications independent of glycemic control could therefore be of considerable value for the management and treatment of diabetes.

A number of metabolic pathways have been implicated as pathophysiologic contributors to microvascular diabetic complications, most notably, increased polyol pathway flux, protein kinase C activation, increased protein glycation leading to formation of advanced glycation end products (AGEs), and hexoamine pathway activation.[2,3] All of these mechanisms, driven by hyperglycemia, are believed to contribute to oxidative-stress mediated tissue damage.

The predicted role for the polyol pathway in the etiology of diabetic complications is well documented.[2,3] The two enzymes in the polyol pathway, aldose reductase (AR), which catalyzes the reduction of glucose to sorbitol, and sorbitol dehydrogenase (SDH), which catalyzes the oxidation of sorbitol to fructose, are abundant in tissues prone to diabetic complications. Under hyperglycemic conditions, increased flux of glucose through the polyol pathway is thought to increase oxidative stress in susceptible tissues, in part via elevating tissue NADH/NAD^+ levels.[4,5]

Selective inhibitors of AR and SDH have been shown to be of potential therapeutic utility in treating diabetic complications in pre-clinical animal models, with beneficial effects on motor nerve conduction velocities, proteinuria, and reduce markers of oxidative stress.[6–9] AR inhibitors have also demonstrated therapeutic potential in clinical studies, improving markers for both neuropathy and nephropathy.[10–13] Some reports have suggested that diabetics have elevated blood fructose or erythrocyte sorbitol levels relative to healthy volunteers, presumably a consequence of elevated flux of glucose through the polyol pathway under hyperglycemic conditions. In fact, AR inhibitors have been demonstrated clinically to reduce erythrocyte sorbitol[14–17] and blood fructose levels.[18,19] However technical and biological variables result in relatively high variability with these biomarkers.

The aim of this study was to investigate the absolute and relative differences in serum fructose and sorbitol measured across three days in healthy volunteers (HV) and patients with type 2 diabetes mellitus (T2DM), looking at sequential samples on each day from both fasting and fed states in subjects under well controlled conditions. A highly accurate and specific gas chromatography-mass spectrometry (GCMS) assay was validated to support this work, and the details of this assay are reported.

Patients and Methods

The study protocol was reviewed by ethical committees and informed consent was obtained from all subjects. Study subjects were confined to a clinical research unit for medical supervision and dietary control for the duration of the study (starting on day −1). HV subjects (n = 13) were free of any dietary supplements or medications for at least 7 days prior to the start of the study. T2DM patients (n = 14; screening HbA_{1c} of 7.5%–10%) treated with 1–2 oral anti-diabetic agents were washed out of these medications for 7 days prior to the start of the study. Serum was collected on three sequential days at −30, −15, and 0 min before breakfast (fasting; after at least a 12 hour fast), and 60 and 120 min after a standardized breakfast (Boost High Protein® at 7 kcal/kg; 55% carbohydrate, 21% fat, 24% protein).

Sample preparation and GCMS analysis were based on the method of Küry and Keller.[20] To 200 µL aliquots of standards (0.025–25 mg/L), QC samples (in human serum), and clinical study samples, 500 ng of fructose and sorbitol internal standards (D-[$U^{13}C_6$] fructose and D-[$U^{13}C_6$] sorbitol) were added. Samples were then deproteinized by $ZnSO_4$—$Ba(OH)_2$ extraction, dried under nitrogen, oximized (500 µL 1% *o*-methylhydroxylamine hydrochloride in pyridine; 2 hr at 70 °C), dried, derivatized (500 µL BSTFA [*N,O*-bis[Trimethylsilyl]trifluoroacetamide]; 20 min at 70 °C), and passed through 0.22 µm PVDF filters into autosampler vials. These samples were analyzed on an Agilent 5973 N Mass Specific Detector (MSD) interfaced with an Agilent 6890 A gas chromatograph equipped with a LEAP Technology GC PAL autosampler with a peltier-cooled sample rack (10 °C). Injection ports and GCMS interface were kept at 250° and 280 °C. Separations were performed on a Varian VF-5 ms fused silica column (30 M × 250 µm × 1 µm

film thickness). Using helium as the carrier gas (0.8 ml/min), derivatized samples (5 µL) were injected at a 50:1 split ratio. The column temp was held at 220 °C for the duration of the 15-minute run. The MSD was run on EI-mode at 70 eV with SIM detection (m/z 307 for fructose, m/z 310 for fructose IS, m/z 319 for sorbitol and m/z 323 for sorbitol IS) for quantification. The MSD was turned off during the elution of glucose peaks.

Results

To accurately quantify sorbitol and fructose in biological samples, a highly sensitive GCMS assay was developed and validated. This assay had excellent intra (0.1%–1.3% CV) and inter-assay (2.9%–14.9%) precision (evaluated with neat, diluted, and spike serum samples) within the range of the standard curve (0.025–25 mg/L) in serum samples. A typical gas chromatography chromatogram for a human serum sample is shown in Figure 1. A peak of varying abundance eluting after the fructose peak was observed, but the identity of this peak was not determined. Based on spiking studies in human serum, we are sure this did not represent myo-inositol, glucose, fructose, or sorbitol. Finally, there was excellent agreement in pilot study samples between serum sorbitol levels and values measured in erythrocytes using this assay, however higher intra-assay variability was seen with analysis of erythrocyte samples.

Using this assay, serum samples from T2DM and HV subjects were analyzed. The two subject populations were well matched for most demographic variables with no significant differences in gender, age, or BMI (Table 1). There were small but statistically significant differences in heart rate and blood pressure, and as expected highly significant differences in fasting blood glucose.

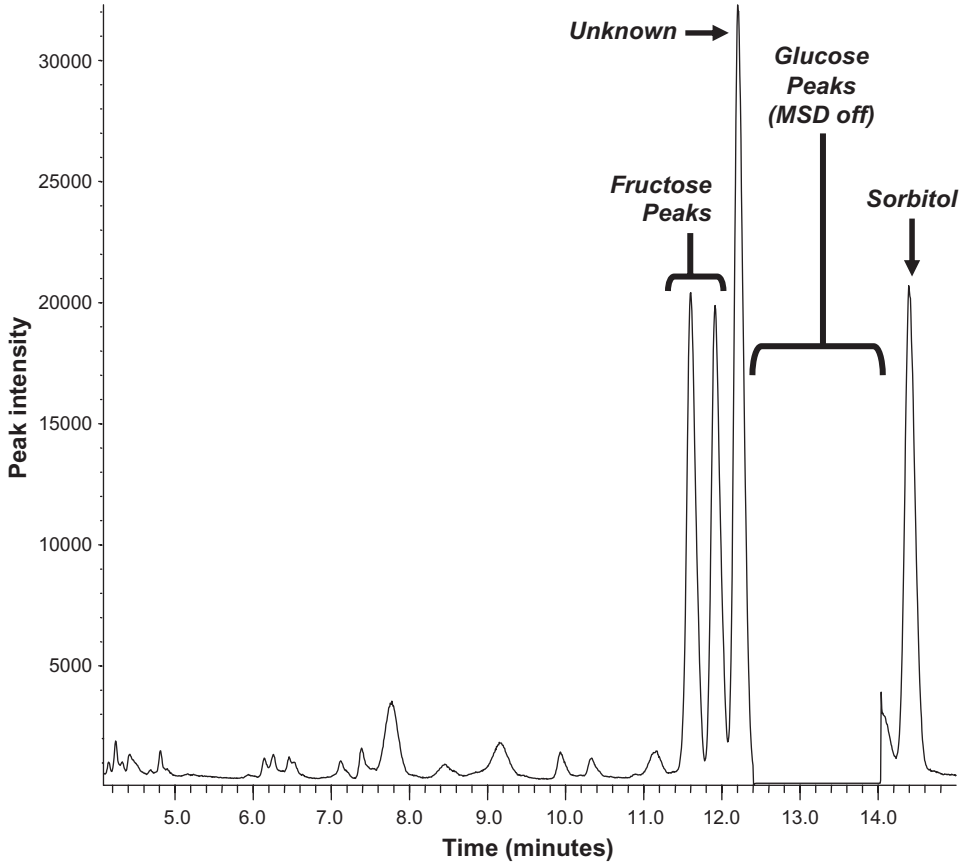

Figure 1. Human serum gas chromatography chromatogram for quantification of fructose and sorbitol.
Typical ion chromatogram of a human serum sample, with the locations of the 2 fructose (at 11.86 and 11.98 min) and 1 sorbitol (at 14.48 min) peaks indicated. The MSD is turned off during elution of the glucose peaks (12.5 to 14.0 min). The identity of the peak following the two fructose peaks was not determined (labeled unknown in the figure), but was unaffected by spiking into samples glucose, sorbitol, fructose, or myo-inositol.

Table 1. Patient characteristics at baseline.[a]

Variable[b]	Healthy volunteers (HV)	Diabetic subjects (T2DM)	*P* value
Age	40.3 (10.1)	46.4 (6.9)	0.086
Gender (M/F)	8/5	7/7	
Ethnicity	6/6/1	2/10/2	
BMI	29.8 (3.5)	32.4 (3.7)	0.066
Heart rate	67.6 (10.2)	75.1 (8.0)	0.044
Systolic BP	116 (7.6)	131 (13)	0.001
Diastolic BP	72.9 (5.2)	78.6 (2.3)	0.047
Fasting glucose	92.5 (6.6)	217 (49)	<0.0001
HbA_{1c}	NA	8.41 (0.80)	

Notes: [a]Reported are the mean (± standard deviation) and *P*-values for all but gender and ethnicity. The data were analyzed using a 2-sample t-test for independent samples. A Levene's test was first conducted to determine if the t-test should be run using equal or unequal variance. HbA_{1c} levels were not measured (NA) in the healthy volunteers. [b]Variable units: age (years); ethnicity (number of white, hispanic, black); BMI ((weight in kilograms)/(height in meters)^2); heart rate (beats per minute); systolic and diastolic blood pressure (mmHg); fasting glucose (mg/dL); HbA_{1c} (%).

Serum fructose and sorbitol levels (in mg/L) from 13 HV and 14 T2DM subjects were measured in blood samples collected on three sequential days from fasting (−30, −15, and 0 min before breakfast) and fed (60 and 120 min after breakfast) blood samples. Fasting and fed intra-subject variability was low (average 21% CV, range 5%–55%, highest for fed samples) so each subjects average levels were calculated. Fasting serum sorbitol levels were significantly higher in T2DM patients than HV subjects, but there was no significant difference in fasting fructose levels (Table 2). Feeding resulted in significant increases in serum fructose and sorbitol, but the magnitude of this increase was much greater for fructose (5.7–6.1 fold increases) than sorbitol (5%–10% increases; potentially as high as a 20% increase with elimination of all data from one T2DM patient that had considerably elevated sorbitol in fasting samples). Postprandial levels of sorbitol but not fructose remained significantly higher in T2DM patients compared to HV subjects. Finally, there was not a significant correlation between serum fructose or sorbitol levels and either heart rate or systolic blood pressure (data not shown). In addition, there did not appear to be a strong correlation between baseline glycemic control marker levels (HbA_{1c}, C-peptide, or fasting glucose) and either sorbitol or fructose in the T2DM patients, however this should not be viewed as definitive due to the limited size of this study.

Table 2. Serum fructose and sorbitol concentrations under fasting and postprandial conditions.[a]

	Healthy volunteers (HV)	Diabetic subjects (T2DM)	*P* value
Serum fructose			
Fasting	1.39 (0.38)	1.48 (0.49)	0.61
Fed	7.98 (2.55)[d]	9.13 (2.29)[d]	0.23
Serum sorbitol			
Fasting	0.164 (0.044)	0.280 (0.163)	0.02[b,c]
Fed	0.181 (0.040)[d]	0.294 (0.064)[d]	<0.0001[b]

Notes: [a]Average fasting and fed serum fructose and sorbitol levels (in mg/L) from 13 HV and 14 T2DM subjects were calculated from measurements of blood samples collected on three sequential days. Reported above are the inter-subject averages (± standard deviation). The data were analyzed using a 2-sample t-test for independent samples, adjusted for equal variance. [b]Based on the observed differences for fructose and sorbitol, and the samples sizes, we have greater than 80% power to detect a 0.1 mg/L difference in sorbitol. [c]Includes data from all subjects. One T2DM patient had considerably elevated fasting serum sorbitol (0.822 ± 0.452 mg/L) in all samples measured, greater than 2.5 fold higher than all other subjects. Fasting and fed fructose levels in this patient were similar to the other subjects in this study; fed sorbitol levels were also higher than for all other individuals, but lower than fasting levels. Deleting the value from this subject resulted in a T2DM fasting sorbitol group mean of 0.238 mg/L (± 0.048), which was also significant greater than the HV fasting sorbitol levels ($P = 0.0004$). [d]$P < 0.002$ in both paired t-test and Wilcoxon signed rank test, comparing fed serum fructose or sorbitol levels to fasting levels (with deletion of data from the T2DM subject with high fasting serum sorbitol noted above).

Discussion

This study shows that serum sorbitol levels, and not fructose, are elevated in fasting serum of T2DM patients with moderately controlled disease (HbA_{1c} 8.41 ± 0.80%, mean ± standard deviation), and that serum sorbitol levels remain significantly elevated with feeding. This is in agreement with studies showing high erythrocyte sorbitol in diabetic patients,[14,17,21,22] and animal models of diabetes.[10,23] In fact, we saw similar magnitude of elevations in serum sorbitol in our T2DM patients (70% higher in fasting samples) as reported with erythrocyte sorbitol content by Asano et al (80% higher).[15] Our data are in contrast to the work of Kawasaki et al[18] who showed fasting serum fructose was increased in diabetic patients. However their study included diabetic patients with poorly controlled disease (HbA_{1c} 10.7 ± 2.4%), for which 2 weeks hospitalization resulted in a reduction in fasting serum fructose to near normal levels, and reductions in other glycemic indicators. Therefore,

the differences in glucose control may at least partially account for the differences in observed fasting serum fructose.

Several AR inhibitors have demonstrated their ability to reduce polyol pathway flux at the target tissue by decreasing sorbitol and/or fructose levels in sensory nerves of diabetic animal models or patients with diabetic neuropathy.[9,12] The potent AR inhibitor fidarestat has also been shown to normalize erythrocyte sorbitol in diabetics.[15] In our study, we observed elevations in serum sorbitol in subjects with T2DM compared to healthy volunteers, and using a mixed-meal tolerance test demonstrated that this elevation is relatively stable in the fed versus fasted state, as compared to the marked changes observed in serum fructose in the post-prandial state. This is consistent with similarly reported relative stability of erythrocyte sorbitol in the fed vs. fasted state.[15] This suggests that sorbitol (serum or erythrocyte) is a more robust marker of polyol pathway flux than fructose, less likely to be influenced by acute changes in metabolic status.

The analytical method for measuring serum fructose and sorbitol levels described in detail in this report was used in a phase 1, multiple dose, clinical study to assess the pharmacodynamic effects of CP-642,931, a potent and selective inhibitor of SDH.[24] In that study, inhibition of SDH in healthy subjects was associated with highly significant dose-dependent increase in serum sorbitol levels, reaching a maximum of a 152-fold increase at 7 days of dosing at 35 mg, the highest dose tested. However no significant changes in fasting serum fructose levels were observed.

The analytical method employed in these studies enables direct and accurate quantification of serum sorbitol and fructose. We have also validated this method for analyses of erythrocyte and urine samples. Furthermore, the method can be easily modified to enable quantification from tissue samples. This will enable further assessment of the relationships between tissue, serum and erythrocyte sorbitol and fructose, and importantly how these markers correlate with glycemic control and efficacy for potent polyol inhibitors developed to reduce or halt the progression of diabetic complications.

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Abbreviations

AR, aldose reductase; SDH, sorbitol dehydroganase; T2DM, type 2 diabetes mellitus; HV, healthy volunteers; GCMS, gas chromatography-mass spectrometry.

Disclosures

This manuscript has been read and approved by all authors. The authors are employees of Pfizer Inc who provided funding for this work. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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