The Natural $^{13}$C Abundance of Plasma Glucose Is a Useful Biomarker of Recent Dietary Caloric Sweetener Intake$^{1,2}$

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Abstract

There is a need for objective biomarkers of dietary intake, because self-reporting is often subject to bias. We tested the validity of a biomarker for the fraction of dietary carbohydrate (CHO) from cane sugar and high fructose corn syrup ($C_4$ sugars) using natural $^{13}$C abundance of plasma glucose. In a randomized, single-blinded, crossover design, 5 participants consumed 3 weight-maintaining diets for 7 d, with a 2-wk washout between diet periods. Diets differed in the fraction of total CHO energy from $C_4$ sugars (5, 16, or 32%). During each diet period, blood samples were drawn at hours 0800 and 1600 on d 1, 3, and 5 and at 0800, 1000, 1200, 1400, and 1600 on d 7. The $\delta^{13}$C abundance of plasma glucose was analyzed via GC-isotope ratio MS. Within each diet period, $\delta^{13}$C abundance of the 0800 fasting glucose did not change from baseline with increasing time during a diet period; however, there was a strong positive correlation ($R^2 = 0.89$) between $\delta^{13}$C abundance of the glucose concentration at 1000 on d 7 and the percent of breakfast CHO from $C_4$ sugars. Also, $\delta^{13}$C abundance of the combined plasma glucose samples on d 7 demonstrated a strong positive correlation ($R^2 = 0.90$) with the percent of total daily CHO from $C_4$ sugars. The natural $\delta^{13}$C abundance of postprandial plasma glucose relative to dietary $C_4$ CHO content was a valid biomarker for contributions of $C_4$ caloric sweeteners from the previous meal. J. Nutr. 140: 333–337, 2010.

Introduction

According to the 2007 USDA Economic Research Service loss-adjusted food availability data (adjusted for spoilage and other waste), mean daily per capita energy available from caloric sweeteners totaled 1960 kJ/d (1). Approximately 99% of these sweeteners are derived from corn [i.e. high fructose corn syrup (HFCS)] and refined sugars (cane and beet sugar). Current research suggests that increased consumption of these caloric sweeteners, typically found in numerous beverages and savory snacks, may contribute to excess energy intake and corresponding body weight gain (2,3). However, van Baak and Astrup (4) have stated in a recent review that there is still insufficient evidence to fully support a relationship between caloric sweetener consumption and obesity.

A potential explanation of this continuing controversy is that current methods to assess dietary intake typically rely on self-reporting methods. Reporting bias would increase variance and possibly obscure a potential relationship (5,6). This issue has been highlighted in recent findings from the Observing Protein and Energy Nutrition Study (7,8), which utilized doubly labeled water as a biomarker of total energy expenditure (9,10) and demonstrated between-individual variation in underreporting. Correspondingly, our goal was to identify an objective biomarker to accurately assess dietary intake of caloric sweeteners. In the case of caloric sweeteners, a biomarker of intake would better assess the relationship between consumption and body weight gain. This biomarker could be used in place of, or as a calibration marker for, self-reporting methods in epidemiological or intervention studies (12,13).

There has been interest in the development of a biomarker of sugar intake. Recently, Joosen et al. (14) have expanded on previous work (15), suggesting 24-h urinary sucrose and fructose concentrations may be used as biomarkers of sugar intake in both normal-weight and obese individuals. Jahren et al. (16) proposed an alternate approach based on the unusual isotopic abundance of $^{13}$C in many sweeteners. Carbon stable isotope abundances may provide a possible biomarker of caloric sweetener intake, because corn and sugar cane plants utilize a unique photosynthetic pathway known as the $C_4$ (Hatch-Slack) pathway to fix atmospheric CO2 into plant tissues (17,18).

Carbon fractionation of $^{13}$C in atmospheric CO2 through the $C_4$ pathway is minimal compared with the more common $C_3$ (Calvin) photosynthetic pathway. As a result, plants utilizing the $C_4$ pathway are isotopically “heavy” with respect to their natural abundance of $^{13}$C relative to plants that utilize the $C_3$ pathway. This contrast in natural $^{13}$C abundance of $C_3$ and $C_4$ plant tissues should persist after they have been consumed. Thus, this unique $^{13}$C signature of caloric sweeteners derived from $C_4$ plants may serve as a possible biomarker of intake.

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The use of natural $^{13}$C abundance signature has been used extensively for food web analysis (19) in both animal ecology (20) and human paleonutrition (21). In the late 1970s, DeNiro and Epstein (22) examined the influence of diet on the natural $^{13}$C abundance of various laboratory animals given controlled diets. They discovered it was possible to perform qualitative dietary analysis based on the natural $^{13}$C abundance of the whole animal carbon. It was concluded that analysis of $^{13}$C abundance of individual tissues or biochemical fractions would best be utilized when dietary carbon is derived from sources with large differences in $^{13}$C abundance (i.e. C3 vs. C4 plants). More recently, diets of laboratory rats have been isotopically manipulated using purified C3 and C4 macronutrients to investigate the routing of dietary carbon to bone collagen biosynthesis (23).

Carbohydrates (CHO) from C4 foods have a mean $^{13}$C of $-11\%_o$, whereas CHO from C3 foods are isotopically “light” with a mean $^{13}$C of $-26\%_o$. We hypothesized that the fraction of dietary CHO from caloric sweeteners could be measured on a binary mixture $^{13}$C abundance scale as follows: 100% caloric sweeteners $= \delta^{13}$C plasma glucose of $-11\%_o$ and 0% caloric sweeteners $= \delta^{13}$C plasma glucose of $-26\%_o$, assuming there is no isotope fractionation during assimilation of the carbon into the body. This indicates a signal of 1.5‰ for each 10% of CHO contributed to an individual’s diet from C4 plant-derived caloric sweeteners. Thus, we conducted a controlled feeding study to test the hypothesis that the natural $^{13}$C abundance of blood glucose reflects the fraction of dietary CHO derived from cane sugar and HFCS (C4 sugars). Blood plasma glucose was selected because it is derived largely from the diet, found in high concentrations in blood, and easily assayed for $^{13}$C abundance.

**Methods**

Participants had no history of metabolic disease, including; diabetes, hypertension, cardiovascular disease, kidney disease, liver disease, or allergies to any of the foods used in the study. The University of Wisconsin-Madison Institutional Review Board approved this study and all participants signed a written informed consent.

Five healthy young adults (3 men, 2 women; age 22 ± 2 y) were fed 3 weight-maintaining diets in a nonrandomized, single-blinded, crossover design. Individual energy requirements for weight maintenance were estimated using the Dietary Reference Intake equations for Estimated Energy Requirements (24). Each diet period lasted 7 consecutive days, with a 2-wk washout between each diet. The diets were formulated by a registered dietitian and were composed of 50% CHO, 30% fat, and 20% protein. The diets differed only in the fraction of total daily CHO derived from cane sugar and HFCS, which equaled 5, 16, and 32% of total CHO/d. The order of diet assignment was: 32, 16, and 5% C4 CHO. The blood sampling protocol is shown in Figure 1. All participants consumed breakfast under supervision at our research kitchen and were provided with lunch, dinner, and snacks to consume on their own. Participants were not required to eat the remaining food items at any preset time, only to eat when hungry, but were instructed to consume all food items at each visit to ensure BW maintenance and as an indirect indicator of dietary compliance. Body weight (kg) did not change during any of the diet periods (mean ± SD; 32% C4 CHO = $-0.1 \pm 0.5$; 16% C4 CHO = $-0.4 \pm 0.3$; 5% C4 CHO = $-0.2 \pm 0.5$).

**Analysis of $^{13}$C abundance of plasma glucose.** Peripheral venous blood was collected into K$_2$ EDTA-coated vacutainers (BD), placed on ice, and centrifuged at 1500 × g for 10 min at 4°C. Following centrifugation, the plasma supernatant was transferred into 2-mL cryovials and stored at −70°C until further analysis. The methods of Remba et al. (25) were used, with slight modifications, for yeast anaerobic fermentation of plasma glucose to CO$_2$ and subsequent analysis via GC-isotope ratio MS. Plasma (250 µL) was added to a 10-mL Exetainer (Labco Limited). Plasma was then treated with 20 µL 5% acetic acid to remove circulating bicarbonate. After vortexing, the sample was evaporated completely under a gentle stream of nitrogen and resuspended in 500 µL deionized water. Exetainers were then capped with screw-top caps containing a rubber septum and flushed with helium gas to provide anaerobic fermentation conditions. Commercially available baker’s yeast (Red Star Yeast) used as a 20 µL yeast/water suspension (5 g/L) was injected via needle and syringe through the rubber septum of the screw-top cap. All tubes were incubated at 30°C overnight for 16 h and subsequently the $^{13}$C abundance was analyzed using a Delta S Isotope Ratio mass spectrometer (Thermo Electron) equipped with a Gas Bench inlet system.

The conventional means for expressing natural abundance isotope ratios is relative to an international standard, expressed as δ‰ units and defined as:

$$\delta^{13}C_{sample} = \frac{\left[\delta^{13}C_{sample} - \delta^{13}C_{standard}\right]}{\left[\delta^{13}C_{standard}\right]} \times 1000$$

From this equation, it can be seen that the δ‰ unit is essentially 10 times the percent difference of the $^{13}$C/$^{12}$C ratio of a sample from the $^{13}$C/$^{12}$C ratio of a standard reference. PeeDee Belemnite Limestone was used as the international standard reference for carbon, $^{13}$C/$^{12}$Cstandard = 0.0112372. A $^{13}$C of 1‰ would correspond to 0.001 atom percent excess. Analytic uncertainty associated with each measurement was ±0.1‰.

The $^{13}$C abundances of all samples were measured against standard CO$_2$ gas (Linde Gas) in the laboratory and expressed relative to PeeDee Belemnite Limestone (26). In addition, isotope analyses were performed by including secondary laboratory standards in each mass spectrometric analysis. The secondary standards were a C$_4$ plant CHO source, sucrose (catalog no. S157, −9.8‰ δ$^{13}$C, American Drug and Chemical) and a C$_3$ plant CHO source, beet sugar (−25.0‰ δ$^{13}$C). ISODAT software (Thermo Electron) was used to perform data processing.

**Statistical analysis.** Results are expressed as means ± SD. Significance was set at α = 0.05. Repeated-measures ANOVA were used to test for differences in δ$^{13}$C of 0800 fasting plasma glucose within each diet period, as well as to test for differences in δ$^{13}$C of plasma glucose as a function of time of each diet period. If the effect of time was significant, differences between means where assessed using Tukey’s multiple comparison test to identify which time points differed with respect to $^{13}$C abundance. Two-sided paired t-tests were used to compare differences between mean δ$^{13}$C of 0800 and 1600 plasma glucose of d 1, 3, and 5 of each diet period. The δ$^{13}$C of d 7 postprandial 1000 plasma glucose was compared by linear (Pearson) regression with the fraction of breakfast CHO derived from C4 sugars. In addition, plasma glucose samples from 1000, 1200, 1400, and 1600 on d 7 were combined and the δ$^{13}$C of this combined d 7 plasma glucose was compared by linear regression with the fraction of total daily CHO coming from C4 sugars. NCSS software was used for statistical analysis.

**Results**

Daily δ$^{13}$C values of 0800 fasting plasma glucose are presented (Table 1). Within each diet period, δ$^{13}$C of 0800 fasting plasma glucose did not change from baseline (d 1) with increasing time during the diet period. Figure 2 shows mean δ$^{13}$C of plasma glucose for the 5 participants on each day during each diet period.
TABLE 1  Plasma glucose 13C abundance in fasting participants who consumed diets containing different levels of total CHO energy from C4 sugars for 7 d1,2

<table>
<thead>
<tr>
<th>C4 CHO/d, %</th>
<th>d 1</th>
<th>d 3</th>
<th>d 5</th>
<th>d 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-26.9 ± 1.6</td>
<td>-25.7 ± 1.9</td>
<td>-27.4 ± 1.1</td>
<td>-26.6 ± 1.5</td>
</tr>
<tr>
<td>16</td>
<td>-26.8 ± 0.8</td>
<td>-26.0 ± 1.1</td>
<td>-26.5 ± 1.1</td>
<td>-25.5 ± 1.0</td>
</tr>
<tr>
<td>32</td>
<td>-26.4 ± 1.9</td>
<td>-25.4 ± 1.3</td>
<td>-25.1 ± 1.1</td>
<td>-25.8 ± 0.8</td>
</tr>
</tbody>
</table>

1 Data are mean ± SD, n = 5.
2 There were no changes in 0800 plasma glucose 13C abundance during each diet period.
3 Diets presented as the fraction of total daily CHO derived from C4 sugars, i.e. cane sugar and HFCS (5, 16, and 32%).

period. During d 1 (Fig. 2A), the 13C of 1600 plasma glucose increased relative to 0800 for all 3 diets (P < 0.05). There was no change in 13C of plasma glucose from 0800 to 1600 on d 3 of any diet. On d 5, the 13C of 1600 plasma glucose again increased compared with the 0800 value for all 3 diets (P < 0.05). During d 7 (Fig. 2B), the 13C of plasma glucose on the 5% C4 CHO diet significantly increased from baseline at 1000, but returned to 0800 levels at 1200, 1400, and 1600. During the 16% C4 CHO diet, there was no change in 13C of plasma glucose at any time point relative to the 0800 baseline. During the 32% C4 CHO diet, as expected, 13C of plasma glucose at all time points significantly increased from 0800 baseline values.

The 13C of 1000 postbreakfast plasma glucose on d 7 was compared by linear regression with the fraction of CHO in the breakfast meal derived from C4 sugars (Fig. 3A), demonstrating a strong positive correlation (R2 = 0.89). The intercept of the regression line, which represents zero caloric sweetener intake, was -26.4‰. This is very similar to the mean 13C abundance of C3 CHO source foods. The slope was 0.16‰ for each 1% increase in the contribution from caloric sweeteners to total CHO intake, which does not differ from the theoretical value for a binary mixture of C3 and C4 carbon sources.

In addition, d 7 plasma glucose samples from 1000, 1200, 1400, and 1600 were combined for each participant to represent each individual’s total pool of plasma glucose during the day (Fig. 3B). The mean 13C abundance of these combined plasma glucose samples was compared by linear regression with the fraction of total CHO/d from C4 sugars, resulting in a strong positive correlation (R2 = 0.90). The intercept of the regression line was -25.9‰, which is again very similar to the mean 13C abundance of C3 CHO source foods. The slope was 0.10‰ for each 1% increase in the contribution from caloric sweeteners to total daily CHO intake. This slope is less than the theoretical value of 0.15‰, suggesting plasma glucose is not solely derived from dietary glucose for these combined sampling times.

**Discussion**

In this study, we demonstrated that the natural 13C abundance of plasma glucose accurately reflected changes in the fraction of dietary CHO derived from cane sugar and HFCS. Specifically, our results demonstrate that the 13C abundance of postprandial plasma glucose was directly proportional to the relative C4 caloric sweetener content of a previous meal (breakfast). This suggests there is little or no isotope fractionation during the initial absorption and metabolism of the glucose and little mixing of carbon from noncarbohydrate sources (23). Thus, this method may be useful for measurement of caloric sweetener intake from a previous meal.

Additionally, our results suggest 13C abundance of combined plasma glucose samples from 1000–1600 were directly
proportional to the relative C₄ caloric sweetener content of the total CHO/d. However, our data indicate that about one-third of the combined plasma glucose from 1000 and 1600 may be derived from alternative sources other than dietary CHO. Indeed, a portion of circulating plasma glucose during the day is derived from gluconeogenesis, as well as liver glycogenolysis (27). The contribution of C₄ carbon to these 2 carbon pools is not as large as those contributing to plasma glucose derived from dietary C₃ CHO sources, which would dilute the natural δ¹³C abundance of circulating plasma glucose. This effectively reduces the utility of using δ¹³C abundance of combined plasma glucose samples from over the course of an eating day to accurately reflect the relative C₄ caloric sweetener content of the total daily CHO.

Despite the positive daytime correlations, this study demonstrates the limitations of using a single overnight fasting plasma glucose sample as the lone biomolecule to assess habitual caloric sweetener intake. The δ¹³C abundance of plasma glucose after an overnight fast was not related to the C₄ sugar content of the diet, as the δ¹³C abundance returned to 0800 baseline levels every morning after an overnight fast. Plasma glucose can arise from 3 sources: absorption from the intestine (diet), glycogenolysis, and gluconeogenesis. The fact that δ¹³C abundance of 0800 fasting plasma glucose does not increase with increasing days of consuming the high-C₄ sugar diet suggests that, of the 2 endogenous glucose-generating pathways, gluconeogenesis is the major source of circulating glucose for the fasting blood specimen. If liver glycogenolysis was the major source, we would expect δ¹³C abundance of fasting plasma glucose to increase, for example, on the high-C₄ sugar diet. With each successive day on this diet, dietary glucose would be synthesized to glycogen during the fed state and would eventually replace preexisting liver glycogen with the isotopically heavier dietary glucose with each cycle of overnight glycogen depletion. Gluconeogenesis, on the other hand, would produce glucose with amino acid-derived carbon, which would not be expected to change with increasing time consuming the diet because we did not systematically manipulate the δ¹³C abundance of dietary protein. The gluconeogenic contribution to circulating glucose after an overnight fast was not unexpected but was considerably larger than expected to the point where the vast majority of circulating glucose appears to come from gluconeogenesis after the overnight fast. Thus, fasting plasma glucose, which is the most common time for blood sampling in most epidemiological studies, fails to reflect habitual caloric sweetener intake.

The lack of plasma glucose samples after 1600 on d 7 may also be viewed as a limitation of our study design. We chose 1600 as the final time point on d 7 for practicality to minimize subject burden of multiple needle sticks. In hindsight, optimal blood sampling would have continued into the evening after the last meal. As previously discussed, however, it is likely that gluconeogenesis attenuation of plasma glucose ¹³C enrichment from dietary sweetener persists into and past the evening meal. Thus, we do not feel that additional evening blood samples would add much to our results.

There was a high degree of between-subject variability in the δ¹³C abundance of fasting plasma glucose regardless of diet. Participants were provided with all study foods but were instructed to eat when hungry to better reflect free-living conditions. A combination of meal timing and the corresponding rate of glucose turnover (i.e. hours) likely contributed to the between-subject variability in δ¹³C abundance on d 7. In addition, C₄ caloric sweeteners were not uniformly distributed across all meals. For example, with the 5% C₄ CHO diet, we did not expect a significant increase in δ¹³C abundance of plasma glucose at any time point, but δ¹³C abundance of 1000 glucose increased relative to baseline on d 7. All C₄ CHO provided during this particular diet period was within the breakfast meal, raising the C₄ fraction of this meal to ~20% of total breakfast CHO. This explains the increase in δ¹³C abundance at 1000, even on a low-C₄ sugar diet, and the corresponding decrease back to baseline levels over the course of the day.

An additional limitation of the δ¹³C abundance of blood glucose as a biomarker of caloric sweetener consumption includes the consumption of corn as a direct food grain. This includes foods containing cornstarches and cornstarch-derived maltodextrins, because this dietary intake cannot be isotopically distinguished from corn carbon intake as a caloric sweetener (HFCS). These foods can change plasma glucose ¹³C enrichment, thereby rendering the proposed method not specific for C₄-derived caloric sweetener intake. Thus, care must be taken in subpopulations that consume large quantities of food grains derived from corn, such as corn tortillas and corn bread. Conversely, beet sugar is a C₃ plant product and this isotopic biomarker method is insensitive to beet sugar sweetener intake. Nonetheless, sweeteners derived from cane sugar and corn (HFCS) contribute ~82% of the total sweetener in use in the US compared with 17% from beet sugar, honey, and maple syrups (1). However, outside of the US and particularly in Europe, beet sugar is a major source of table sugar. Thus, ¹³C abundance of blood glucose as a biomarker of caloric sweetener consumption would be subject to error in the European population.

It should be noted that an alternate approach to this isotopic biomarker has been suggested, using bulk carbon analysis of blood, hair, or nails (28). This would avoid the temporal limitation of blood glucose discussed above; however, most of the carbon in blood and virtually all of the carbon in hair or nails is in the form of protein, largely derived from dietary protein (23). Dietary protein is derived, in turn, from animal and plant protein. Animal protein is also marked with C₄ carbon because of the use of corn for animal feed in the US (29). Thus, the bulk ¹³C abundance would be heavily influenced by the isotopic abundance of dietary protein. Protein-based specimens are therefore not likely to be of value for the assessment of caloric sweetener intake, because they would be confounded by animal product consumption.

In conclusion, this method is useful for the measurement of caloric sweetener intake from the previous meal. Further investigations are needed to identify a biomolecule that integrates the ¹³C signature from caloric sweeteners over a period of days or weeks to better represent usual intakes, which would increase the utility of this biomarker method for epidemiological studies. Dilution of ¹³C abundance of overnight fasting plasma glucose by gluconeogenesis limits the value of this biomarker method.

Acknowledgments
D.A.S. designed research; Q-Y.L. recruited participants and organized data collection; C.M.C. performed isotopic analyses; A.L.A. was the registered dietician and prepared all study meals; C.M.C. and D.A.S. wrote the paper. All authors read and approved the final version of the paper.

Literature Cited