A Convenient LC-MS Method for Assessment of Glucose Kinetics In Vivo with \(^{\text{d}-}[^{13}\text{C}_6]\)Glucose as a Tracer

Haoyue Zhang,\(^1\) Robert D. Stevens,\(^2\) Sarah P. Young,\(^1\) Richard Surwit,\(^3\) Anastasia Georgiades,\(^3\) Raymond Boston,\(^4\) and David S. Millington\(^1\)*

BACKGROUND: The isotope-labeled intravenous glucose tolerance test (IVGTT) combined with computer modeling is widely used to derive parameters related to glucose metabolism in vivo. Most of these methods involve use of either \(^2\text{H}_2\)-labeled or \(^{13}\text{C}_1\)-labeled \(\text{d}\)-glucose as a tracer with GC-MS to measure the isotope enrichment. These methods are challenging, both technologically and economically. We have developed a novel approach that is suitable for labeled-IVGTT studies involving a large cohort of individuals.

METHODS: The tracer, \(\text{d-}\left[^{13}\text{C}_6\right]\text{glucose}\), is a low-cost alternative with the significant advantage that the sixth isotope of natural glucose has virtually zero natural abundance, which facilitates isotopomer analysis with \(<1\%\) labeled glucose in the infusate. After deproteinization of plasma samples collected at various times, glucose is converted to a stable derivative, purified by solid-phase extraction (SPE), and analyzed by HPLC–electrospray ionization mass spectrometry to accumulate the isotope-abundance data for the \(A+2, A+3,\) and \(A+6\) ions of the glucose derivative. A 2-pool modeling program was used to derive standard kinetic parameters.

RESULTS: With labeled-IVGTT data from 10 healthy male individuals, the values for insulin sensitivity, glucose effectiveness, and the plasma clearance rate estimated with the 2-pool minimal model compared well with values obtained via traditional methods.

CONCLUSIONS: The relative simplicity and robustness of the new method permit the preparation and analysis of up to 48 samples/day, a throughput equivalent to 2 complete IVGTT experiments, and this method is readily adaptable to existing 96 well–format purification and analytical systems.

By challenging the endocrine system with energy substrates such as glucose, we are able to appreciate how effectively the counterregulatory forces of the system are able to restore its basal state. Through the use of the intravenous glucose tolerance test (IVGTT)\(^5\) in conjunction with modeling methods, investigators have shown that circulating glucose (below the renal threshold) is cleared via the action of 2 distinct forces, glucose-mediated glucose control (glucose effectiveness) and insulin-mediated glucose disposal (insulin sensitivity) \((1)\). Furthermore, pathophysiological and behavioral conditions may manifest themselves through impairment of one or both of these processes. Thus, it is critically important that methods are available for reliably estimating these 2 forces.

Studies have shown that the application of the single-compartment minimal model to the sampled-glucose challenge for estimating the processes known as glucose-mediated glucose disposal and insulin sensitivity may affect the accuracy and precision of such methods. The labeled IVGTT is a powerful, noninvasive method for quantifying indices of glucose metabolism with the 2-pool glucose (TPG) model, also called the 2-compartment minimal model \((2, 3)\). In the TPG model, the glucose distribution volume is divided into an accessible, rapidly equilibrating pool and a slowly equilibrating exchangeable pool that is assumed to be insulin sensitive.

This model is considered to represent the true physiological state much more closely than the single-compartment model. The use of labeled glucose to assess metabolism in vivo permits separation of the

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1 Department of Pediatrics, Medical Genetics Division, 2 Stedman Center for Nutrition, and 3 Department of Psychiatry and Behavioral Sciences, Medical Psychology Division, Duke University Medical Center, Durham, NC; 4 School of Veterinary Medicine, University of Pennsylvania, Kennet Square, PA.

* Address correspondence to this author at: DUMC Biochemical Genetics Laboratory, 801 Capitol Drive, Suite 6, Durham, NC 27713. Fax 919-549-0709; e-mail dmsli@duke.edu.

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insulin effect on glucose disposal from the suppression of hepatic glucose production by the principle of tracer-to-tracer indistinguishability. The traditional analytical method for measuring isotope enrichment is GC-MS with selected ion monitoring after converting glucose to a stable derivative, such as the pentaacetate or $\alpha$-D-glucorufuronic cyclic 1,2,3,5-bis(butylboronate)-6-acetate (4). Typically, if $^2$H$_2$-labeled glucose is used, at least 8% of it is required in the glucose bolus for IVGTT to enable accurate measurement of isotope ratios. It is possible to reduce the required amount of stable isotope by 15-fold (essentially to tracer concentrations) through the use of D-$^{13}$C$_6$glucose as a tracer and gas chromatography–combustion–isotope ratio mass spectrometry to measure isotopic enrichment (5, 6). The specialized equipment required is not widely available, however; an economically viable alternative is to use D-$^{13}$C$_6$glucose as a tracer, which both minimizes the impact of possible carbon recycling and enables a reduction in the tracer amount to <1% of the IVGTT bolus and the use of standard analytical equipment.

Instead of GC-MS, we adopted a novel approach that uses HPLC–electrospray ionization mass spectrometry (HPLC-ESI-MS), which is fast becoming standard equipment in clinical diagnostic and research laboratories. This method requires the formation of a suitable derivative for ESI. The reaction of glucose and other reducing sugars to form Schiff base derivatives is well documented (7), and the derivative formed by the reaction with butyl-p-aminobenzoate and sodium cyanoborohydride (butyl-PABA) has both chemical stability and excellent properties for ESI (8, 9). We have successfully used this method with HPLC and HPLC-ESI-MS to analyze glucose tetrasaccharides in blood and urine (10, 11).

We therefore reasoned that this method would facilitate a high sample throughput while maintaining sufficient accuracy and precision for the kinetic modeling. We report the details of this method and the results of its application to a cohort of nondiabetic participants in a clinical trial. We discuss its advantages relative to the characteristics of previously published methods. Healthy white men participated in this study, and we used $^{13}$C$_6$-labeled IVGTT along with a computer program coupled with database technology, AKA-TPG (12, 13), to estimate the kinetic parameters of glucose effectiveness, insulin sensitivity, and plasma clearance rate.

Materials and Methods

REAGENTS AND CHEMICALS
Deionized water was collected from an in-house system (PureFlow Technologies). Burdick & Jackson–brand methanol was purchased from VWR. D-$^{13}$C$_6$Glucose was obtained from Cambridge Isotope Laboratories. Butyl-PABA, sodium cyanoborohydride, and glacial acetic acid were purchased from Sigma–Aldrich. Polystyrene microtiter plates (96-well) were from Evergreen Scientific, and 1.5-mL, 11-mm borosilicate vials and 11-mm caps with Teflon/rubber linings were from Sun-SRI/Fisher Thermo Scientific.

The working reagent for derivatization of up to 50 samples was prepared as needed by mixing 0.385 g butyl-PABA, 0.625 g sodium cyanoborohydride, 0.375 mL glacial acetic acid, and 4.63 mL methanol. The quantities were proportionally smaller for fewer samples.

EQUIPMENT
Solid-phase extraction (SPE) Sep-Pak Vac C18 cartridges (1 mL, 100 mg) were from Waters Corporation, and the HPLC column was a Supelcosil™ LC-18 (33 mm $\times$ 4.6 mm, 3-$\mu$m particle size). The guard column used was a 2 cm $\times$ 4 mm Supelcosil™ LC-18 Supelguard cartridge. A Waters Acquity UPLC® (ultra performance liquid chromatography) BEH C18 column (2.1 mm $\times$ 50 mm, 1.7-$\mu$m particle size) was used for experiments performed on the Acquity UPLC system. A Visiprep multiple-SPE cartridge eluter (Sigma–Aldrich) was used for sample preparation. Analyses were performed on a Waters Quattro Micro triple-quadrupole mass spectrometer equipped with an ESI source, a CTC autosampler (Agilent Technologies), and an 1100 LC pump (Agilent Technologies), or on a similar system equipped with an Acquity solvent system and autosampler (Waters).

STUDY PARTICIPANTS AND LABELED-IVGTT PROTOCOL
The study participants were healthy white adult males (n = 10) who consented for the labeled-IVGTT study under an institutional review board–approved protocol. The IVGTT was performed essentially as previously described (1, 2). In brief, a bolus of 300 mg/kg n-glucose plus 2 mg/kg D-$^{13}$C$_6$glucose was infused in vivo over a period of approximately 1 min, and 3-mL blood samples were collected into purple-top tubes (with EDTA as the anticoagulant) from a separate line at times −1, 2, 3, 4, 5, 6, 10, 12, 15, 20, 30, 40, 60, 80, 100, 120, 140, 150, 180, 210, and 240 min, relative to the time of bolus administration (i.e., 0 min). Plasma was collected after centrifugation. Samples were assayed for glucose and insulin in the Duke Clinical Laboratory, and a separate 1-mL aliquot of each sample was retained for isotope analysis by MS. Plasma samples were kept frozen at −70 °C until analysis.
SAMPLE PREPARATION FOR ANALYSIS

Vials containing plasma samples for analysis were thawed and vortex-mixed. A 100-µL aliquot was transferred to a separate microcentrifuge tube. The sample was deproteinized by adding 500 µL methanol, vortex-mixing for 20 s, and centrifugation at 2000g for 5 min. The supernatant (500 µL) was transferred to 1.5-mL borosilicate vials and evaporated under nitrogen at 50 °C. The dried extracts were reconstituted in 20 µL deionized water, and 100 µL of the derivatization reagent (see above) was added. The vials were capped, incubated in an oven for 1 h at 80 °C, removed, and allowed to cool.

The equipment at our disposal (Supelco Visiprep) permitted convenient sample purification in batches of 12. The SPE cartridges were primed with 1 mL methanol, reprimed with 1 mL water, and reequilibrated with the sample-transfer matrix (0.2 mL of acetonitrile: water; volume ratio, 15:85). The 12 vials were uncapped, and each reaction mixture was quenched with approximately 900 µL of the sample-transfer matrix. The content of each vial was transferred with a glass Pasteur pipette to an SPE cartridge. The cartridges were washed twice with 1 mL of the transfer matrix and eluted with 1 mL acetonitrile:water (volume ratio, 30: 70). Even with this crude apparatus, a single technician was comfortably able to process and analyze up 48 samples/day, which is equivalent to 2 complete IVGTT studies, plus appropriate controls. The eluates were mixed for 20 s, and centrifugation at 2000 g approximately 4 min. The HPLC was operated in the isocratic mode, and the analysis time for each sample was approximately 4 min.

MS ANALYSIS

We analyzed the plasma glucose derivatives by HPLC-ESI-MS with a Waters Quattro Micro triple-quadrupole mass spectrometer, which was operated in single-quadrupole selected ion monitoring mode. We used the following MS parameter values: capillary voltage, 3 kV; cone voltage, 60 V; extractor, 3 V; RF lens, 0.2 V; source temperature, 100 °C; desolvation temperature, 200 °C; desolvation gas flow, 250 L/h. The resolving quadrupole was set at unit mass resolution.

The derivatized-glucose isotope signals corresponding to A + 2 (m/z 360), A + 3 (m/z 361) and A + 6 (m/z 364) were collected by selected ion recording during the elution profile, in which A was the most abundant isotope signal of the protonated derivative (m/z 358). The selection of A + 2 as the glucose signal was based on its comparable order-of-magnitude intensity with the A + 3 signal and the A + 6 signal at peak values.

The A + 3 signal was monitored to check for possible recycling of the D-[13C6]glucose.

The D-[13C6]glucose concentration in each sample was calculated as follows:

\[
g^* = \frac{G_i \times ([A + 6]/[A + 2]) \times 100}{([A + 6]/[A + 2]) \times 100 + 3759.39},
\]

where \(g^*\) and \(G_i\) are the D-[13C6]glucose concentration (calculated) and the total glucose concentration (measured at time \(t\)), respectively, in millimoles per liter (superscript asterisk denotes tracer-related variables that include the metabolism indices in this report); \([A + 2]\) and \([A + 6]\) are the absolute intensities of the monitored isotopes of the glucose derivative; and 3759.39 is the theoretical sum of the peak intensities of A, A +1, A +2, A +3, A +4, A +5 in natural abundance normalized to the A + 2 peak intensity (arbitrarily 100). We made the reasonable assumption that the natural abundance of A +6 is zero.

Results

HPLC-MS AND UPLC-MS ANALYSIS

Fig. 1 shows selected HPLC-ESI-MS and UPLC-ESI-MS ion current chromatograms for the A + 6 and A + 2 isotopes of the protonated molecular ion ([M + H]⁺) cluster for a healthy individual at times 0 min and 5 min. The D-[13C6]glucose value (A + 6 isotope) at baseline was essentially zero and was markedly increased, close to its peak value, at 5 min. The A + 6 isotope signal was still clearly discernible even 240 min after infusion (data not shown). The retention time on HPLC-MS was 2.46 min, and the cycle time for the analysis was 4.05 min. Most of the data described in this report were generated with these conditions.

The recent acquisition of a UPLC system prompted us to compare its features for this analysis. The peak intensities achieved by UPLC were approximately 5 times greater than those obtained with HPLC because of improved resolution and the reduced retention time of 0.7 min (Fig. 1). Although not essential for the analysis, UPLC clearly reduces the analysis time and reduces the imprecision of the method.

GLUCOSE DISPOSAL AND ENDOGENOUS GLUCOSE

Fig. 2 shows a typical D-[13C6]glucose clearance curve during IVGTT measured by the isotope-abundance method. For most of the participants, the D-[13C6]glucose concentration started to decrease approximately 10 min after infusion and continued until 240 min. Labeled-glucose concentrations did not reach baseline concentration, even at the end of the IVGTT time course. The CV for these measurements was de-
terminated by replicate analysis \((n = 10)\) and was \(<0.6\%\) until at least 40 min, when it began an increase to about 1.7\% by 240 min. These errors are much lower than those reported for other methods, probably because of the use of the \(^{13}\text{C}_6\) isotope, which has negligible natural abundance in unlabeled glucose.

Fig. 2 also shows a plot of the endogenous glucose concentration at each time point in the same individual, which was calculated as follows:

\[
G_e(t) = G_t(t) - 151g^*(t),
\]

where \(G_e(t)\), \(G_t(t)\), and \(g(t)\) are the concentrations of endogenous glucose, total blood glucose, and labeled glucose, respectively, at time \(t\), and \(151g^*(t)\) is the sum of the infused unlabeled glucose and labeled glucose in the bloodstream at time \(t\) estimated from the bolus ratio. The endogenous concentration of glucose decreased as expected after glucose infusion, started to recover at 100 min, but did not recover completely to the baseline concentration, even after 240 min. These data reflect the changes in hepatic glucose production during the IVGTT.

PARAMETERS ESTIMATED WITH THE 2-POOL MODEL
We used a TPG modeling program to estimate several parameters (insulin sensitivity, glucose effectiveness, glucose plasma clearance rate) for the 10 white male study participants (Table 1) \((13)\).

Table 2 compares these parameter values with those reported for healthy white males. The differences are relatively minor and may be due to differences in diet and other genetic and environmental factors.

GLUCOSE RECYCLING
One of the attributes of the selected label, \(\text{d}-[^{13}\text{C}_6]\text{glucose}\), is that if it is recycled via metabolism in the liver, the only detectable product should be \(\text{d}-[^{13}\text{C}_3]\text{glucose}\). Theoretically, the possibility of \(\text{d}-[^{13}\text{C}_6]\text{glucose}\) being reformed via the Cori cycle is negligible \((<0.002\%)\). Glucose recycling was estimated from the change in the \([A + 3]/[A + 2]\) ratio. During the IVGTT, this ratio remained essentially constant, and observed changes were well within the experimental error of the measurement. The suppression of hepatic glucose production during IVGTT, as shown in Fig. 2, may account for a lack of evidence for recycling in the first part of the experiment, but it is unlikely to affect the latter stages. We therefore conclude that recycling, if it occurred at all, was undetectable by this method.
Discussion

The isotope-labeled IVGTT with 2-pool modeling is a widely accepted tool for studying glucose kinetics in vivo. The objective of the new methods we have described was to simplify the analytical protocol while improving analytical sensitivity, so as to facilitate studies of cohorts of individuals larger than have previously been studied. In particular, we were interested in studying relationships between glucose metabolism and a psychological parameter, hostility. To that end, we have performed the labeled IVGTT with a cohort of 130 individuals recruited from different ethnic groups and both sexes. The relatively large number of individuals and the relatively high volume of samples generated from the IVGTT procedure prompted the development of a robust, straightforward, and economical method. The use of \( \text{D-}[13\text{C}_6] \) glucose allowed us to perform labeled IVGTT on a person of average body weight (80 kg) with only 0.16 g of the isotope tracer diluted with 24 g of unlabeled glucose. Because the cost of this tracer is less than US$14 per patient, cost is no longer an important factor limiting this type of study. The ability of MS to detect this tracer at a proportion of \( \% < 1 \) in the infusate, which is further diluted in the body, is greatly facilitated by the lack of a perceptible endogenous signal for \( \text{D-}[13\text{C}_6] \) glucose at baseline.

We reasoned that the use of published methods to analyze more than 20 samples from each of >130 patients would be challenging for a laboratory that does not have a dedicated research GC-MS system; therefore, we developed a new method that uses LC-ESI-MS instrumentation, which is fast becoming standard equipment in clinical research laboratories. Attempts to analyze glucose directly by HPLC-ESI-MS were unsatisfactory (results not shown), but its conversion to...

Table 1. Metabolic indices from 10 healthy white men estimated with the TPG model.a

<table>
<thead>
<tr>
<th>Study individual</th>
<th>( S_{I^2*,10^2}\cdot \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \cdot \mu\text{U}^{-1} \cdot \text{mL} )</th>
<th>( S_{G^{<em>2}</em>} ), ( \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
<th>PCR, ( \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.9</td>
<td>1.4</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>6.7</td>
<td>1.1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>18.9</td>
<td>1.4</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>17.4</td>
<td>1.2</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>16.3</td>
<td>1.1</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>25.2</td>
<td>1.8</td>
<td>3.4</td>
</tr>
<tr>
<td>7</td>
<td>36.4</td>
<td>2.2</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>28.6</td>
<td>1.6</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>33.8</td>
<td>1.9</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>18.6</td>
<td>2.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Mean (SE)</td>
<td>22.4 (2.8)</td>
<td>1.6 (0.2)</td>
<td>3.0 (0.3)</td>
</tr>
</tbody>
</table>

* \( S_{I^2*} \), 2-pool insulin sensitivity; \( S_{G^{*2}*} \), 2-pool glucose effectiveness; PCR, plasma clearance rate.

Table 2. Comparison of metabolic parameter values estimated with the TPG model in different studies.a

<table>
<thead>
<tr>
<th>Reference</th>
<th>( S_{I^2*,10^2}\cdot \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \cdot \mu\text{U}^{-1} \cdot \text{mL} )</th>
<th>( S_{G^{<em>2}</em>} ), ( \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
<th>PCR, ( \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vicini et al. (3)(^b)</td>
<td>13.83 (2.54)</td>
<td>0.85 (0.14)</td>
<td>2.02 (0.14)</td>
</tr>
<tr>
<td>Nishida et al. (14)(^c)</td>
<td>11.9 (2.4)</td>
<td>0.60 (0.05)</td>
<td>1.6 (0.1)</td>
</tr>
<tr>
<td>Nishida et al. (14)(^d)</td>
<td>24.6 (3.0)</td>
<td>0.81 (0.08)</td>
<td>2.4 (0.6)</td>
</tr>
<tr>
<td>Boston et al. (13)(^e)</td>
<td>18 (1)</td>
<td>1.19 (0.04)</td>
<td>2.2 (0.8)</td>
</tr>
<tr>
<td>This study (healthy males)</td>
<td>22.4 (2.8)</td>
<td>1.6 (0.2)</td>
<td>3.0 (0.3)</td>
</tr>
</tbody>
</table>

* Data are presented as the mean (SE). \( S_{I^2*} \), 2-pool insulin sensitivity; \( S_{G^{*2}*} \), 2-pool glucose effectiveness; PCR, plasma clearance rate.

\( ^b \) A bolus of glucose enriched with either \([6,6-2\text{H}_2]\)glucose or \([2-2\text{H}]\)glucose was administered to 14 young adults.

\( ^c \) A bolus of \([6,6-2\text{H}_2]\)glucose was administered to 12 middle-aged, sedentary men.

\( ^d \) Middle-aged trained men.

\( ^e \) Data are for 15 healthy, young Nordic men.

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the butyl-PABA derivative, which is performed in a single step, afforded a derivative with excellent characteristics for HPLC-ESI-MS. The butyl-PABA derivative is also chemically stable, and samples that were stored at −20 °C for more than 2 years could still be analyzed successfully. Although the derivatized product must be purified by SPE before analysis to remove excess reagent and salts, the procedure described in this report is amenable to existing 96-well plate technology, which will facilitate high sample throughputs. Compared with existing methods, the convenience of this new method should greatly facilitate any study of glucose kinetics, not only labeled-IVGTT studies like the one we have described.

Our measurements of minimal 2-pool modeling parameters for a cohort of 10 white males compare well with the results described in previously published reports (Table 2), indicating that the new method appears to have introduced no notable bias. The CVs of the stable-isotope measurements were <2% at all time points. The ability to achieve this degree of imprecision is largely due to the absence of an endogenous signal for D-[13C6]glucose.

References