Development of a LC/MS method to determine [66-2H] glucose enrichments in human plasma samples

Kristin H. Bourret

The University of Montana

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Development of a LC/MS method to determine [6,6-\(^2\)H] glucose enrichments in human plasma samples.

By

Kristin H. Bourret

B.S. University of Montana, 1999

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For the degree of

Master of Science

The University of Montana

2001

Approved by:

[Signature]

Chairperson

[Signature]

Dean, Graduate School

9-18-01
Method development for the quantitation of glucose and [6,6-²H] glucose in human plasma samples using LC/MS.

The purpose of this research was to develop a method that accurately detects the enrichment of [6,6-²H] glucose in human plasma samples via LC/MS. Traditionally, the analysis of [6,6-²H] glucose has been conducted using GC/MS. The benefit to a LC/MS method is the avoidance of derivatizing glucose for analysis as is required with GC/MS. 

**Method:** Plasma samples were acquired from five female subjects participating in an exercise study. Each subject performed a 60-minute cycle ergometry exercise bout while being infused with [6,6-²H] glucose. Additional plasma was obtained from a previously conducted exercise study, which acquired data by GC/MS. Three different ionization techniques were explored; negative ion electrospray (ESI) LC/MS, negative ion atmospheric pressure chemical ionization (APCI) LC/MS, and positive ion ESI LC/MS.

**Results:** The data with the best signal, peak resolution, and signal to noise ratio were acquired using positive ion ESI LC/MS. Data did not match previously measured enrichments by GC/MS and predicted trends were not exhibited by the freshly acquired plasma samples. Accurate results were obtained when analyzing glucose standards mixed in DDI water and spiked with 1-5% [6,6-²H] glucose ($R^2 = 0.989$). On the contrary, data resulting from glucose standards again spiked 1-5% with [6,6-²H] glucose but that were from plasma showed no discernible trends ($R^2 = 0.187$).

**Discussion:** The problem with the analysis of [6,6-²H] glucose was unique to samples coming from plasma. The isotopic peaks were larger than predicted for background samples and for samples drawn after the infusion started. A contaminant molecule with the same molecular weight as the isotope was suspected. Sorbitol, a reduced form of glucose has the molecular weight of 182 amu and was pursued as a candidate. Glucose standards in DDI water spiked with sorbitol showed a ten-fold increase in the measurement of theoretical enrichments ($R^2 = 0.998$).

**Conclusion:** Further research is needed in the development of a LC/MS method. Techniques for better separation of glucose from contaminant molecules should be employed. The use of carbohydrate LC columns specifically made to separate monosacharrides may improve separation, as may the use of a quadrupole mass analyzer.
Acknowledgments

First, thanks to Brent Ruby, Steve Gaskill, and Gene Burns, for the passion you bring to your work, the HHP department, and for all the students you have and will continue to inspire. Brent, Steve, Dusty, and Kent, thanks for your early morning help on weekends and holidays.

I would not be on my way to the great north without the extraordinary help from the Thompson group. Thanks for contributing your knowledge, time, enthusiasm and commitment to this project. To Travis, the craziest chemist I know, the energy you bring to the lab is infectious. Thanks for showing me around and giving me a home in the pharmacy department. Every Thursday evening in AK I’ll raise a pint to you. To Doug, the mass spec master, it was supposed to be easy!!! However, as you often pointed out, easy is not interesting. Next time there is a decade gap in the literature I will take note, thinking long and hard before I decide to repeat a method that has not been used since. Thanks for all of your effort! I wish you the best of luck with your fly-fishing, guitar playing, and of course mass spec endeavors. To Dr. Chuck Thompson, you truly are one of a kind. It has been an honor working with you. Thanks for being a part of my committee, providing me with direction and structure, and for all the time you spent reviewing and commenting on my work.

Thanks to Missoula for providing a place to work and play hard for the last six years. The friendly people, beautiful trails, and excellent nightlife will be missed.

To all the folks at MESSI, thanks for providing me with a colorful job, great friends, never ending adventures on the streets of Missoula and of course financial and mental instability. You’ve made life exciting for the past three years.

Finally, thanks to my wacky friends especially my roommates. I’m grateful to you all for putting up with late nights, early mornings, and unplanned parties. May we all get more sleep!

Best of luck to all! Onward and upward we shall go.
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Chapter One: Introduction

In 1959 Steele established a method for measuring the turnover rate and size of the body's glucose pool using a stable isotope (Steele, 1959). This method has been widely used in the field of exercise science to measure the appearance rate of metabolic substrates found in the blood. Steele’s method has since been modified by Wolfe and is utilized today by researchers when measuring glucose kinetics in vivo (Wolfe, 1992). Metabolic research using [6,6-²H] glucose has become increasingly important to the field of exercise science and especially carbohydrate feeding studies. Quantitation of glucose metabolism involves the use of stable and/or radioactive isotopes. In order to limit the risk incurred by human subjects, stable isotopes are employed to avoid any ill effects of radiation. The use of [6,6-²H] glucose enables researchers to assign relative proportions of carbohydrate oxidation rates to either glycogen stores or blood glucose.

An understanding of glucose metabolism is needed in order to provide accurate recommendations on nutrition, training, and performance for competitive athletes. Glucose kinetics is of particular importance to the endurance athlete who may deplete carbohydrate stores over the course of an event and must rely heavily on exogenous sources of glucose. Performance and rate of recovery may be enhanced with proper carbohydrate intake before, during, and after competition (Febbraio et al., 2000). A better understanding of glucose metabolism will not only benefit athletes but also those with various medical conditions or job related environmental stresses. People working in very hot or cold ambient temperatures, patients suffering from thermal injuries, and
pregnant women face conditions that alter energy expenditure and thus energy intake. The elucidation of nutritional requirements for these populations as well as athletes may be aided by metabolism studies that utilize [6,6-^H] glucose.

Analysis of [6,6-^H] glucose can lead to the determination of relative blood glucose and stored glycogen oxidation rates. This requires the use of a mass spectrometer (MS), which evolved from work done in 1912 by J.J. Thompson (Siuzdak, 1996). MS provides information on a molecule’s molecular weight and/or structure. In exercise physiology, mass spectroscopy has been used for the analysis of isotope data gathered from metabolic studies. The MS detects the enrichment of an isotopic tracer molecule within the tracee pool. It is this enrichment that enables the quantitation of a particular metabolite’s disposal and uptake rates. Traditionally, metabolic studies using [6,6-^H] glucose have used gas-chromatography mass spectrometry (GC/MS) (Febbraio et al, 2000, Marmy-Conus et al., 1996, Freidlander et al., 1998, Zderic et al., 2001). The GC/MS is a gas chromatographic system that uses a mass spectrometer as a detector. The GC separates compounds that are thermally stable, chemically inert, and volatile at temperatures generally below 300° C (James et al., 1952). The sample is vaporized and carried through a column by an inert gas, usually helium. Most substances within the body are not thermally stable, chemically inert or volatile.

The five OH functional groups in hexoses like glucose produce strong intermolecular forces that decrease the molecules ability to vaporize as required for the GC. Therefore, glucose must be made into a derivative for GC/MS analysis. Derivatization masks the OH groups and increases the molecules volatility, which
facilitates GC/MS analysis. The pentaacetyl derivative of glucose is used for GC/MS analysis. Assumptions must be made that all five of the OH functional groups acquire an acetyl group with 100% conversion (Wolfe, 1992). If one of the five functional groups is not converted, the MS will not detect the molecule as glucose, possibly lowering sensitivity. This presents an appealing challenge to find a way to analyze glucose in its native, underivatized form.

James McLafferty developed liquid-chromatography mass spectrometry in 1973 (Siuzdak, 1996), seventeen years after the advent of GC/MS. This kind of interfacing with the mass spectrometer does not require the derivatization of glucose and is, therefore, more suitable for analyzing biological substrates. Currently, there are several common LC interfacing techniques available. These techniques differ in their optimal analyte, ionization type, and maximum solvent flow rates (Chapman, 1998). The evolution of electrospray ionization (ESI) has facilitated the use of LC/MS methods for polar molecule analysis, negating the need for derivatization (Chapman, 1998 and Gelpi 1995). ESI is accomplished by charging the sample liquid in a narrow-bore metal capillary. Small charged droplets are formed. Eventually desorption of the sample occurs from the droplets and it is sent into the mass spectrometer. ESI is the best interfacing candidate for establishing a method to analyze glucose from plasma samples because of its mild ionization and low thermal input.

Reid et al., 1990 proposed a methodology in 1990 using thermospray LC/MS to analyze enrichments of \([1-^2H_1]\) glucose in human plasma samples. To date, this is the only LC/MS method used for the analysis of plasma glucose enrichments. The method
uses negative ion thermospray LC/MS to measure the enrichment of $[1^{-2}\text{H}_1]$ glucose in plasma samples. Since this work, electrospray (ESI) LC/MS has replaced thermospray and revolutionized biomolecular analysis. The purpose of our research is to develop a LC/MS method for the determination of carbohydrate utilization patterns.
Problem

To develop a method for the evaluation of [6,6-^H] glucose enrichments using LC/MS. This study seeks to determine optimal sample prep, LC, and mass spectrometer conditions for proper analysis. The data provided will help determine if LC/MS is an appropriate method for this type of analysis.

Research Hypothesis

Hypothesis One

Plasma glucose enrichments using [6,6-^H] glucose can be determined using LC/MS.

Justification

LC/MS is an appealing technique for glucose enrichment analysis since glucose derivatization is not necessary.

Hypothesis Two

The results obtained by LC/MS should mirror results obtained via GC/MS.

Justification

Both GC/MS and LC/MS require the same purification steps with the exception of derivatization. Assuming this step does not profoundly change the amount of glucose in each sample, LC/MS should detect similar enrichments.

Significance of the Study
There is widespread use of the [6,6-^H] glucose isotope in metabolic research. To our knowledge GC/MS is the only method utilized in these studies. This method requires the addition of an acetyl functional group to all five of the OH functional groups of glucose. Only one published study has used LC/MS to find enrichments for plasma glucose and no studies have been published using ESI LC/MS for the [6,6-^H] glucose isotope.

**Rationale for the Study**

The development of an LC/MS protocol for the analysis of plasma glucose will complement existing methods of analysis, offering the possibility of examining multiple analytes, and permit high-throughput to researchers with the option of using LC/MS. Analyzing glucose in its native form eliminates concern over derivatization and artifact production.

**Limitations**

1/ Sensitivity. TOF MS is not as sensitive as a quadrupole MS for single ion monitoring (SIM) conditions.

2/ Sample prep. Plasma samples were thawed and refrozen several times throughout the course of analysis. To avoid degradation of samples, they were only thawed for the time needed to collect sample and immediately refrozen. Additionally, once purified samples were transferred into auto sampler vials they were kept frozen in –80°C freezer.
Plasma samples. A limited volume of plasma was available to perform experiments. Old samples from a previous study collected four years prior to this research were used during method development.

Definition of Terms

**LC/MS.** Liquid-chromatography mass spectrometry refers to a liquid chromatography system with a mass spectrometer as a detector.

**GC/MS.** Gas-chromatography mass spectrometry refers to a gas chromatography system with a mass spectrometer as a detector.

**Tracer.** Labeled metabolite infused to study a metabolite of interest.

**Tracee.** The metabolite whose kinetics are being examined.

**Ra.** The rate of appearance of a given metabolite into the circulation.

**Rd.** The rate of disposal of a given metabolite out of the circulation.

**Isotopic enrichment.** The ratio of tracer to tracee determined with liquid chromatography-mass spectrometry. Used to calculate Ra and Rd.

**[6,6-²H] Glucose.** A glucose molecule in which the two hydrogens on carbon six have been replaced with deuterium. This molecule is stable and used to measure glucose kinetics in vivo.
Chapter Two: Methodology

Materials and Methods

Human Subjects

Plasma samples were obtained from five recreationally active female students from the University of Montana campus. Subjects were 18-23 years old and volunteered for this study. Subjects completed consent forms (Appendix) and were made aware of the rigor of the study as well as risks involved with the procedures. Subjects reported to the lab for two tracer trials once during the mid follicular and once during the mid luteal phase of their menstrual cycle. The University of Montana Institutional Review Board (IRB) and Human Subjects Committee approved the study (Appendix).

Body composition

Hydrostatic weighing with a digital scale (Exertech, Dresbach, MN) at residual lung volume was used to calculate percent body fat and fat free mass. The residual lung volume was calculated using a Helium dilution technique using calculations developed by Goldman and Becklace. Calculations for percent body fat utilized body density and age/gender specific equations (Lohman, 1992).

Exercise tests

Subjects were instructed to refrain from the ingestion of nicotine, caffeine, alcohol, and food 10 hours prior to exercise trials and to refrain from exercise 15 hours before exercise.
testing. Subjects reported to the lab for peak VO\(_2\) (volume of oxygen consumed)/ lactate threshold determination one week to one month before experimental trials began. All exercise tests were conducted on a Monark cycle ergometer (Model 824E). A modified TrueMax 2400 Parvo-medics (Salt Lake City, UT) metabolic system was used to analyze expired oxygen and carbon dioxide concentrations. The system was equipped with a Hans Rudolph (Hans Rudolph, Inc., Kansas City, MO) medium-flow (10-120 L min\(^{-1}\)) pneumotach along with Beckman oxygen and carbon dioxide analyzers. Prior to each test the metabolic system was calibrated with known concentrations of O\(_2\) and CO\(_2\) (15.2% and 5.17%). The system was recalibrated if gases were not within 5.0% of known values. Flow rate was calibrated using a 3L syringe per manufacturer’s instructions (Parvo-medics Operators Manual, 1999). During testing, heart rate was continuously monitored using a telemetry chest strap and monitor (Polar, Port Washington, NY).

**Peak VO\(_2\) / Lactate Threshold cycle test**

The first lab visit was a peak VO\(_2\)/ lactate threshold test. Prior to the test a venous catheter was placed in an antecubital vein for blood sampling throughout exercise. In order to prescribe exercise intensities, blood samples were collected for lactate threshold determination. VCO\(_2\), VO\(_2\) and respiratory exchange ratio (RER) were collected every 20 seconds. The protocol consisted of a 5-minute warm-up followed by 2-minute stages starting at 58.8 W and increased approximately 10 W each stage until volitional exhaustion. Peak VO\(_2\) was defined as reaching two of the following three criteria: an RER value above 1.0, a plateau in VO\(_2\), and a HR greater that 95% of predicted maximal
HR. Blood samples of 1-2 ml were drawn the last 20 seconds of each 2 minute stage. The test was stopped when the subject met two of the three peak VO$_2$ criteria. Immediately after each sample was collected, 0.5 ml of whole blood was added to a test tube containing 1 ml of chilled 7% perchloric acid and frozen at -20 °C until samples were thawed and centrifuged. The supernatant was removed and lactate concentrations were later determined using a spectrophotometric method. The estimated lactate threshold power output was determined as the last workload before a nonlinear increase in blood lactate concentration. The highest recorded heart rate was reported as the subject’s maximal heart rate.

Experimental Trial
Subjects reported to the lab at 6:00 a.m., approximately 10 hours fasted. Indwelling catheters (18-20 gauge Teflon) were placed in an antecubital vein of each arm and kept patent with a continuous saline drip (0.45% sodium chloride). A background sample of 4 ml was drawn before a primed constant infusion of the isotope [6,6-$^3$H] glucose (Cambridge Isotope Laboratories, Andover, MA) was started. The 10-minute priming dose was 4.0 μmol/kg min$^{-1}$, which was followed by a 50-minute constant infusion at a rate of 0.40 μmol/kg min$^{-1}$ in order to reach isotopic equilibrium. A Harvard infusion pump (Cambridge Isotopes Laboratories, Woburn, MA) depressed a 60-ml syringe containing the isotope. Next, an oral glucose bolus of 2.0 g of glucose / kg fat free mass (FFM) (Fisher) was given in a 22% solution. The subject remained sedentary for 20 minutes before beginning the exercise session at 90% of lactate threshold on a cycle
ergometer. Blood samples were taken every 5-10 minutes throughout exercise (see timeline).

Analysis

Blood sampling timeline:

<table>
<thead>
<tr>
<th>Priming dose 4.0 µmol/kg min⁻¹</th>
<th>Constant infusion 0.40 µmol/kg min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>-60</td>
<td>CHO Drink</td>
</tr>
<tr>
<td>-2</td>
<td>0 10 20 25 30 40 50 60</td>
</tr>
</tbody>
</table>

Figure 2.1. Total blood volume drawn = 65 ml, each sample will contain from 4-10 ml + 1.5 ml discard.

Blood samples were collected in EDTA treated tubes for the analysis of glucose and in plain glass tubes containing chilled 7% perchloric acid for plasma lactate. Glucose and lactate concentrations were analyzed using an enzymatic (Sigma) spectrophotometric technique (Milton Roy Spectronic 401). Samples drawn for each timepoint were analyzed for plasma glucose and samples drawn at time 0, 30, and 60 of exercise were analyzed for plasma lactate. Samples from each subject were analyzed together in duplicate.
**Hardware and Reagents**

Polypropylene test tubes with cap, 12 x 75 mm were obtained from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Zinc sulphate (0.3 N, 14-4) barium hydroxide (0.3 N, 14-3) and mixed-bed ion exchange resin (TMD-8) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). [6,6-^H] Glucose was obtained from Cambridge Isotope Laboratories (Andover, Massachusetts, USA). D-Glucose (anhydrous) was obtained from Mallinckrodt Baker, Inc. (Paris, KT, USA).

**Apparatus**

High performance liquid chromatography/mass spectroscopy (HPLC/MS) was carried out using a MicroMass LCT mass spectrometer running in the positive ion mode. The HPLC was performed with a Waters 2790 pump on a Symmetry C$_{18}$ (3.5 μm, 2.1 x 50 mm i.d.) (Waters Corporation, Milford, Massachusetts, USA), 20 μl injection with a flow rate of 1ml/min (2/98 acetonitrile: water). Glucose and [6,6-^H] glucose were observed as their sodiated species yielding mass spectral peaks corresponding to [M+Na]$^+$ of 203 (180 + 23) and 205 (182 + 23), respectively.

The mass spectrometer was equipped with the electrospray source with the settings: capillary voltage = 3200 V; sample cone = 10 V; extraction cone = 5 V; ESI desolvation temperature = 400 °C; source temperature = 150 °C; desolvation gas flow ~ 650 l/hr.
**Figure 2.2.** Glucose isolation and purification from obtaining subjects blood to LC/MS analysis.
Sample preparation

Plasma serum samples (500 µL) were added to 12 x 75 mm test tubes and deproteinized by the addition of barium hydroxide (0.3 N, 750 µL) and zinc sulphate (0.3M, 750 µL), vortexed and stored on ice for 20 minutes. The tubes were then transferred to the speed vac centrifuge and spun at maximum speed for 20 minutes in a cold room at 4 °C. The tubes were removed and the supernatant was added to a mixed-bed ion exchange column (3 mL disposable syringe, filled to 1.5 mL, Becton Dickinson and Company, Franklin Lakes, NJ) and the column was washed five times (400 µL) with double de-ionized (DDI) water (2500 µL, total volume). The resultant solution was lyophilized and the residue was reconstituted in DDI water (500 µL), transferred to an HPLC auto sampler vial and kept frozen at −80 °C until HPLC/MS analysis. The thawed, vortexed samples were injected into the HPLC/MS (20 µL) and the mass spectral peaks corresponding to 203 and 205 were monitored. The ratio of 205 to 203 was calculated for each time point.

Standards

Two sets of standard solutions were prepared, one in water and the other in plasma. Stock solutions of [6,6-²H] glucose (Cambridge Isotope Laboratories, Andover MA) and D-glucose (Mallinckrodt Baker, Inc., Paris, KT) at 5 mM concentrations were prepared in DDI water. Serial dilutions were made at 1%, 2%, 3%, 4%, and 5% [6,6-²H]
glucose enrichments. A second set of standard stock solutions were mixed. A 10 mM [6,6-²H] glucose solution was added to aliquots of a 6.97 mM plasma sample. Again dilutions were made and plasma samples were spiked from 1-5% with [6,6-²H] glucose.

Analysis of Standards

The above standard solutions were prepared to determine the accuracy of the LC/MS values. Analysis of standards was conducted in a manner that would also assess the effect of the sample prep protocol as expressed in the procedure below.

Procedure:

The blank (100 µl, 1%, 2%, 3%, 4%, and 5% standards in water) were transferred to HPLC auto sampler vial and analyzed before any sample prep occurred. Deproteinizing agents were added to the remaining solution as stated in sample prep section but with volumes adjusted, put one ice for 20 minutes, centrifuge in the cold room for 15 minutes, and the supernatant was transferred into HPLC auto sampler vials for analysis. With 250 µl of the above standards the full sample prep (deproteinization, chromatography and lyophilization) was conducted before analysis. Because proteins in the plasma may clog the HPLC sampler, spiked plasma standards were only analyzed after the deproteinizing step and after complete prep.

Statistical Procedures

1) Glucose (pre exercise 0, 10, 20, 30, 40, 50, and 60 minutes of exercise) and lactate (time 0, 30, and 60 of exercise) concentrations were determined using a SuperAnova
statistical package (Abacus, Inc, Berkeley, CA). The level of significance was set at an overall experimental alpha of 0.05.

2) Descriptive variables VO2 peak (ml·kg⁻¹·min⁻¹) and % VO2 peak at lactate threshold were reported expressed as mean plus or minus standard error.
Chapter Three: Results and Discussion

Subject characteristics

Plasma samples were taken from five recreationally active females tested during the follicular and luteal phases. The physical characteristics are reported in Table 1. None of the subjects were using oral contraceptives and all were menstruating normally for the six months prior to the study.

Table 3.1. Physical characteristics of female subjects. Expressed as mean ± standard deviation (n = 5).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>20 ± 1.7</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>166.8 ± 6.9</td>
</tr>
<tr>
<td>Body Mass (kg)</td>
<td>64.69 ± 12.7</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>19.94 ± 4.3</td>
</tr>
<tr>
<td>Maximum Heart Rate (bpm)</td>
<td>193 ± 7</td>
</tr>
<tr>
<td>VO₂ peak (l/min)</td>
<td>3.07 ± 0.5</td>
</tr>
<tr>
<td>VO₂ peak (ml · kg⁻¹/min)</td>
<td>47.84 ± 4.4</td>
</tr>
<tr>
<td>% VO₂ peak @ lactate threshold</td>
<td>63.10 ± 5.0</td>
</tr>
</tbody>
</table>
Workload Intensities during exercise trials

The intended exercise intensity was 90% VO\textsubscript{2} at the lactate threshold (% VO\textsubscript{2} @ LT).

No differences were found with respect to workload intensity during the 60-minute exercise trials.

Table 3.2. Workload intensities for the exercise trial reported expressed as mean $\pm$ standard deviation (n = 5). Early refers to 1-30 minutes of exercise and late represents 31-60 minutes of exercise during each trial.

<table>
<thead>
<tr>
<th></th>
<th>Follicular %VO\textsubscript{2} peak (ml/kg/min)</th>
<th>Luteal %VO\textsubscript{2} peak (ml/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td></td>
<td>Late</td>
<td>Late</td>
</tr>
<tr>
<td>59.11 $\pm$ 4.45</td>
<td>58.86 $\pm$ 4.84</td>
<td>57.72 $\pm$ 4.84</td>
</tr>
</tbody>
</table>

Plasma glucose and lactate concentrations

There were significant differences in plasma glucose concentrations at time 0 and 10 of exercise after the oral bolus (2.0 g / kg FFM) was given. By minute 20 of exercise, plasma glucose concentrations were normalized. Subjects demonstrated an excellent ability of normalizing blood glucose concentrations after such a large load. Plasma lactate was determined for time 0, 30, and 60 minutes of exercise. There were no significant differences between the menstrual phases but a main effect for time is
indicated in Figure 3.2. Significant differences exist between time 0 and both exercising
time points (30 and 60).

* Figure 3.1. Plasma glucose response due to oral glucose load given 20 minutes before
exercise (n = 5).
Figure 3.2. Plasma lactate concentrations illustrating a main effect for time at time 30 and 60 of exercise (n = 5).

**Substrate Oxidation**

As indicated by Figure 3.3, subjects oxidized more carbohydrate during the luteal phase than follicular phase. Previous research in our lab (Zderic et al., 2001) found the opposite trend, reporting increased fat oxidation in the luteal phase, associated with elevated levels of estradiol and progesterone. The only differences between the previous study and the present were the carbohydrate drink before exercise and a longer duration of exercise at the chosen intensity.
Figure 3.3. Contribution of fat and carbohydrate to substrate oxidation (1 = follicular, 2 = luteal).
**Plasma prep for LC/MS analysis**

Ba(OH)$_2$ and ZnSO$_4$ were added to plasma samples to precipitate out proteins. The samples were centrifuged and the supernatant was added to mixed-bed ion exchange columns so that molecules other than glucose would be removed increasing the purity of each sample. Each column was washed with DDI water and then lyophilized. Samples were reconstituted in DDI water with the same volume of the original plasma sample.

**Ionization techniques explored**

*Negative ion electrospray LC/MS*

The first technique utilized to detect glucose and [6,6-$^2$H] glucose was negative ion ESI-LC/MS. Glucose, with a mass to charge ratio (m/z) of 179 was observed with decent signal intensity (Fig 3.4). However, the isotopic peak at m/z 181 sits in the background noise (Fig 3.5). Areas under this peak were not quantitated because of the large signal to noise ratio. When comparing spectra from the front and back end of the total ion chromatogram, the 181 peak was shifted slightly as indicated in Figure 3.6. The tracer tracee ratio from the front end of the peak was generally smaller than that calculated from the back end. This was consistently observed with plasma samples analyzed in the negative ion ESI mode. These observations led to the exploration of other ionization techniques and the question of whether or not other molecules were co-eluting at the isotopic peak.
Figure 3.4. Total ion chromatogram (TIC) in negative ion ESI.

Figure 3.5. Background noise in negative ion ESI.
Figure 3.6. Mass shift illustrated in negative ion ESI. The top (0.439) peak represents a scan from the back end of the TIC and the bottom represents a scan from the front of peak (0.356).

**Negative ion APCI**

The next method explored was negative ion atmospheric pressure chemical ionization (APCI). With this method, initial data showed improved signal intensity, lower background and improved peak resolution (Fig 3.7 and 3.8). All samples from the exercise study were analyzed using this method looking at peaks 181/179 and the chlorine-associated glucose at 217/215. Chlorinate-associated peaks were analyzed because they consistently appeared in the spectra and both peaks were well above the background noise. The 217 and 215 peaks were promising prospects to quantitate enrichments because their signal intensities were not as dramatically different as the 179
and 181 peaks. Slight differences in the tracer peak intensity did not change the tracer tracee ratio (TTR) as drastically. But with either set of peaks tracer tracee ratios were higher than predicted especially for background samples (Table 3.3). Additionally, when enrichments were plotted the data did not illustrate any discernible trends (Fig 3.9). The high background values produced negative enrichments for time points occurring after the tracer infusion started. When correcting for background values (taking theoretical background values) there was still no observable trend. Enrichments are predicted to stay constant over the course of exercise but with a possible drop towards the end. These trends were not observed.

**Figure 3.7.** Improved background noise in negative ion APCI.
Figure 3.8. Peak resolution in negative ion APCI.

Table 3.3. Representative subject's tracer tracee ratio (TTR) and enrichment (E) for glucose (181/179) and glucose + chlorine (217/215). E = sample TTR - background TTR.

<table>
<thead>
<tr>
<th></th>
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Figure 3.9. Time vs. enrichment for a representative subject in the exercise study. Enrichments for 181/179 and 217/215 peaks are shown.

Chromatography

Chromatography was experimented with while searching for better separation of glucose from other molecules. The purpose of running the samples through a LC column was to increase purity and decrease the chance for a co-elutant at the tracer peak. Flow rates, size of the column, and solvent conditions were varied (water, acetonitrile, and ammonium acetate). Unfortunately, running the samples through the column did not
improve background, separation, or ionization. Without any improvement in the data another ionization method was investigated and loop injections were made.

*Positive ion electrospray [M+Na] 205/203*

Through communication with another laboratory it was recommended to look for the sodiated peak [M + Na] of glucose using positive ion ESI. The peaks appeared at m/z 203 and 205 with good signal intensity. This form of ionization (cationization) is well suited for carbohydrates because of the electronegativity associated with their hydroxyl functional groups. Standard solutions made up in double deionized water were prepared to test the instrumentation before more plasma was used from the exercise study. Glucose enriched 1-5% with [6,6 $^2$H$_2$] glucose was run through the mass spectrometer (Fig 3.10). Actual enrichments were plotted against theoretical values. The mass spectrometer accurately measured the enrichments for the standards in water as indicated by the standard curve ($R^2 = 0.9888$) (Fig 3.15). The accuracy of the instrument led to the belief the method would also measure plasma samples correctly. Therefore, two sets of plasma samples from the exercise study subjects were prepared and analyzed for enrichment. Again the enrichments did not indicate the predicted trend and the samples that were previously analyzed via GC/MS did not match LC/MS results (Table 3.4 and Fig 3.16). Until this stage of method development, it was thought problems with the data were due to the ionization method. In order to elucidate why the plasma samples were not accurately detected, a set of spiked plasma standards were prepared as described in
the methodology section (Fig 3.11, 3.12 and 3.13). When analyzing the spiked plasma standards a poor correlation was observed between theoretical values and experimental values ($R^2 = 0.187$) (Fig 3.16). It became clear that there was a problem seeing the isotope peak in only the plasma samples. An additional observation with the plasma standards was that a slight mass shift with the isotopic glucose peak was again detected as with negative ion ESI. This indicates the possibility of another molecule co-eluting with [6,6 $^2$H] glucose. The intriguing point about the two sets of standards was the inaccuracy of detecting enrichments for glucose that came from plasma. The enrichment values for both blank and enriched plasma samples indicate a molecule with the same molecular weight that is artificially inflating these values.

![Figure 3.10](image)

**Figure 3.10.** Positive ion ESI mode illustrating a 5mM standard solution in water (on the bottom with the theoretical spectra on top).
**Figure 3.11.** Positive ion ESI mode showing a total ion chromatogram of a 2% standard solution prepared from human plasma.

**Figure 3.12.** Positive ion ESI mode illustrating the integration of background plasma sample a 205 to 203 ratio of 5.08%.
Figure 3.13. Positive ion ESI mode representing 5% standard solution prepared from human plasma. Spectrum indicates an enrichment of approximately 10%.
Table 3.4. Comparison of enrichments obtained by LC/MS and GC/MS from previous exercise study (Tracer tracee ratio = 205/203 x 100).

**Previously analyzed samples**

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Figure 3.14. GC/MS vs LC/MS enrichment data.
Figure 3.15. Standard curve for glucose spiked with 1-5% [6,6$^2$H] glucose.
Figure 3.16. Standard curve for plasma samples spiked with 1-5% [6,6\textsuperscript{2}H] glucose.

Sorbitol Experiments

Sorbitol, a reduced form of glucose, is a candidate for a tracer peak contaminant with a molecular weight of approximately 182.17 amu. Sorbitol would not interfere with GC/MS analysis, as it would form a hexaacetate derivative instead of the hexaacetate derivative that glucose forms. Figure 3.17 shows a spectrum of sorbitol dissolved in DDI water at m/z 205, the same mass as [6,6\textsuperscript{-2}H] glucose. Both glucose and sorbitol peracetate derivatives were analyzed via LC/MS (m/z 413 and 457 respectively) and are shown in Figure 3.18 representing the large mass difference that occurs after derivatization. The
glucose samples that were spiked with sorbitol are represented by a standard curve shown in Figure 3.19 ($R^2 = 0.9982$). As indicated by the graph, the experimental values are approximately ten times the theoretical values. It appears that sorbitol demonstrates a much stronger (~10 times) interaction with sodium than glucose. For some reason the $[M+Na]^+$ is more likely to form with sorbitol than with glucose. If sorbitol is in the plasma, even in small amounts, it may contaminate the isotopic glucose peak, making the peak appear larger than predicted. Equal amounts of glucose and sorbitol were mixed in DDI water and analyzed to confirm sorbitol’s increased ability to form $[M+Na]^+$ when compared to glucose. If both molecules form the ion at the same rate, then similar peak intensities would are predicted. The results show a 205 peak that is much larger than the glucose peak formed at 203 (Fig 3.20) practically drowning out the glucose peak at 203.

A blank plasma sample was derivitized in an attempt to identify sorbitol within a plasma sample by its derivative m/z 457. The sample was prepared as described in the methodology but instead of reconstituting in DDI water, it was reconstituted in 2:1 acetic anhydride and pyridine to form the glucose and possibly the sorbitol peracetate derivatives. The sorbitol derivative was formed (Fig 3.21). Even small amounts of sorbitol may have a large effect on tracer peak quantitation as evidenced by the standard curve (Fig 3.19). Sorbitol in trace amounts become magnified, skewing the enrichments of [6,6-$^2$H] glucose. A final experiment conducted involved the addition of equal amounts of glucose pentaacetate and sorbitol hexaacetate to DDI water. A similar trend was observed as occurred with the native forms of both molecules but the results were not
as drastic (Fig 3.22). The derivatization has illustrated sorbitol contained in the plasma. Additionally, the hexaacetate derivative of sorbitol is formed but does not interfere with isotopic peak calculations for the determination of enrichment. In order to identify the contaminant molecule as sorbitol, retention times on a LC column should be analyzed. If sorbitol elutes at the same time as glucose this would be convincing evidence that we have targeted the molecule responsible for skewing enrichment data.

**Figure 3.17.** Spectrum of Sorbitol m/z 205.
Figure 3.18. Spectrum of glucose pentaacetate (m/z 413) and sorbitol hexaacetate (m/z 457).
Figure 3.19. 5 mM glucose solution spiked 1-5 % with Sorbitol.
**Figure 3.20.** Equal amounts of glucose and sorbitol in DDI water.

**Figure 3.21.** Sorbitol hexaacetate detected in background plasma sample.
Figure 3.22. Equal amounts of glucose pentaacetate and sorbitol hexaacetate in DDI water.
Conclusion

The results suggest that of the three ionization techniques explored, positive ion ESI LC/MS produced the best data with respect to the signal to noise and signal intensity. Standards prepared in water were accurately measured however, standards prepared from plasma were not. The separation of glucose from other molecules in plasma is not achieved by the sample preparation and LC/MS techniques. Therefore, this LC/MS method is inadequate at determining the enrichment of [6,6-\(^2\)H] glucose in plasma. Further research to elucidate the identity of a contaminant molecule and the development of a more effective separation technique is needed. This research should investigate using a quadrupole analyzer, which may increase the instrument's sensitivity. Also, the use of carbohydrate LC columns made to separate monosaccharides will likely enhance glucose separation. Although ESI LC/MS has revolutionized many areas of scientific research a definitive method for the determination of isotopic glucose enrichment remains elusive.
PAR-Q & YOU

(A Questionnaire for People Aged 15 to 89)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read each question carefully and answer each one honestly. Choose YES or NO.

YES  NO
1. Have you had heart disease or any other condition that would normally make you limit your physical activity? ☐ ☐
2. Have you had a heart attack or stroke in the past year? ☐ ☐
3. Do you have angina (chest pain) when you do physical activity? ☐ ☐
4. Do you have high blood pressure? ☐ ☐
5. Do you have diabetes? ☐ ☐
6. Do you have a knee or joint problem that could be made worse by a change in your physical activity? ☐ ☐
7. Are you taking any medications that could interfere with the changes you are planning to make? ☐ ☐

If you answered YES to one or more of these questions, consult your doctor before you make any changes in your physical activity.

If you answered NO to all questions, you may proceed as described in the PAR-Q guidelines but if you have any concerns you should discuss these with your doctor.

You are encouraged to copy the PAR-Q but only if you use the entire form.

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Personal Data Sheet

Name ________________________________
Address ________________________________
Phone Number(s) ________________________________

Age ____________

In case of an emergency, contact (closest relative)
Name ________________________________
Relation ________________________________
Phone ________________________________
Address ________________________________

Menstrual History
1. Have you had a normal period for at least the last 6 months?

2. What is your history using oral birth control (what kind, how long)?

3. When was your last period?

4. How many days is your cycle (20, 30)?

Exercise History
1. Describe the type of exercise (duration, intensity and frequency) you participate in.
A menstrual phase comparison in eumenorrheic females: glucose kinetics during submaximal exercise in response to an oral glucose load - Research

STUDY DIRECTOR(S): Kristin Bourret (406) 542-1296 University of Montana
Brent Ruby, Ph.D. (406) 243-2117 University of Montana
Steven Gaskill, Ph.D. (406) 243-4268 University of Montana

This consent form may contain words that are new to you. If you read any words that are not clear to you, please ask the person who gave you this form to explain them to you.

The purpose of this investigation is to determine if the menstrual cycle may alter which muscle fuel (carbohydrate and fat) I use during submaximal exercise. The information collected from this study should help determine if females have different fuel requirements during each phase of the menstrual cycle while performing exercise.

Participation in this study will include 1) pre-screening questionnaires to determine health and menstrual history as well as exercise training habits (~30 minutes), 2) a measure of percent body fat obtained by measuring my body weight underwater (~1 hour), 3) a measure of my leg volume obtained by measuring leg circumferences, leg length, and leg width (~20 minutes), 3) a cycling test to determine my body's maximal ability to consume oxygen and at what intensity my anaerobic threshold occurs (~1 hour), 4) two 60-minute cycling trials that will involve the infusion of about 1 gram of a glucose stable isotope (~3.5 hours each totaling 7 hours). The total time commitment is estimated at ~9 hours and 50 minutes spread out over 4 laboratory visits.

Following the completion of the pre-screening questionnaires my leg volume and body composition will be measured. Two different measures are necessary to calculate my body composition (the amount of muscle and fat on your body): 1) helium breathing technique to determine the amount of trapped air in my lungs after I exhale maximally, and 2) underwater weighing. This procedure will require that I be seated in a large tank of water and weighed when completely submerged with the air completely exhaled from my lungs (I will be underwater for approximately 15 seconds per trial). For this trial, I am to bring a bathing suit and towel. This procedure will take approximately one hour total.
Leg volume measurements will be taken to estimate my leg muscle size. This uses a skinfold technique, similar to what is frequently used in health clubs to estimate body composition. The technique uses a caliper to measure specific folds of skin in different areas of my leg. Additionally circumferences and leg diameter will be measured with a tape measure. Leg volume measurements will take approximately 20 minutes.

The cycling maximal oxygen consumption/anaerobic threshold test will require me to work at increasing resistance until near exhaustion. The test will require that I have a venous catheter inserted in my arm and remain there for the duration of the test. Blood samples will be obtained every two minutes (approximately ½ teaspoon each, total estimate 5-6 teaspoons). I will breathe into a mouthpiece to monitor how much oxygen I am using. This test will take approximately 1 hour.

The 60-minute cycling trial will require me to report to the Human Performance Lab early in the morning (~5AM). Indwelling venous catheters will be inserted into one vein in each arm for blood sampling and infusion of a glucose stable isotope during rest and cycling. I will rest on a bed for approximately 100 minutes with catheters in place before the cycling trial. After 100 minutes I will drink a solution containing glucose in a concentration similar to soda (22%), then I will continue to rest for 30 additional minutes before starting exercise. The cycling trial will consist of riding an exercise bike for 60 minutes at a moderately hard intensity. This test will require that I breathe into a mouthpiece to monitor oxygen use during exercise. This test will take approximately 3.5 hours. Blood samples will be obtained every 5 minutes during exercise and three times before exercise totaling approximately 2.5-3.0 fluid ounces (~75-80 mL). Transportation will be provided for me.

I will not exercise during the 15 hours prior to any test and will not consume any food for 10 hours prior to each test (with the exception of body composition testing). I will also provide a list of foods consumed in the previous 48 hours before the 30-minute cycling trial. I will report to the Human Performance Laboratory in room #121 in McGill Hall for all of my testing.

It is expected that I will have minimal discomfort as a result of my participation in this study. Nausea and the possibility of vomiting may occur in response to the oral glucose load. The graded cycle ergometry test has certain risks associated with it including loss of consciousness and stroke (0.05% of the time), heart attack and death (0.005% of the time) or musculoskeletal injury or muscle soreness. Although the risks associated with these tests are minimal, all testing will be conducted under the guidelines for exercise testing observed by the American College of Sports Medicine. Blood sampling (venopuncture) can sometimes be associated with risks of bruising (10%), infection (less than 1%) and clotting problems (less than 1%). All of these risks will be minimized by the use of sterile procedures and trained technicians. There are no known risks associated with the presence of stable isotopes in the body, as they are naturally present.

All exercise testing and blood sampling will be supervised and conducted by Brent C. Ruby, Ph.D. an Exercise Physiologist and trained phlebotomist and his trained staff of assistants. In the event you are injured while participating in this research study, you
should contact Dr. Ruby in the lab (406-243-2117) or at home (406-542-2513). All results will be kept in strict confidence among the subject involved and the Principal Investigators and other Co-investigators. During the entire period of data collection, subject records will be kept within the Human Performance Laboratory and will be locked under the direction of the Principal Investigator.

BENEFITS OF PARTICIPATING IN THIS STUDY
1. There is no promise that you will receive any benefit from taking part in this study.
2. You will receive information concerning body composition that may be compared with norms for your age, sex and sport. You will also receive information on your maximal aerobic capacity and anaerobic threshold, which you may compare with norms for your age, sex and sport. There are no other direct benefits to the participants in the study.

CONFIDENTIALITY
1. Your records will be kept private and will not be released without your consent except as required by law.
2. Only the researcher and his faculty supervisor will have access to the files.
3. Your identity will be kept confidential.
4. If the results of this study are written in a scientific journal or presented at a scientific meeting, your name will not be used.
5. All data, identified only by an anonymous ID #, will be stored in our laboratory.
6. Your signed consent form and information sheet will be stored in a locked office separate from the data.

COMPENSATION FOR INJURY
Although we believe that the risk of taking part in this study is minimal, the following liability statement is required in all University of Montana consent forms. “In the event that you are injured as a result of this research you should individually seek appropriate medical treatment. If the injury is caused by negligence of the University or any of its employees, you may be entitled to reimbursement pursuant to the Comprehensive State Insurance Plan established by the Department of Administration under the authority of M.C.A., Title 2, Chapter 9. In the event of a claim for such injury, further information may be obtained from the University’s Claim representative or University Legal Counsel.”

VOLUNTARY PARTICIPATION/withdrawal
1. You have the right to request that a test be stopped at any time.
2. Your decision to take part in this research study is entirely voluntary.
3. You may refuse to take part in or you may withdraw from the study at any time without penalty or loss of benefits to which you are normally entitled.
4. You may leave the study for any reason.

You may be asked to leave the study for any of the following reasons:
1. Failure to follow the study investigator’s instructions.
2. A serious adverse reaction, which may require evaluation.
3. The study director/investigator thinks it is in the best interest of your health and welfare.
4. The study is terminated.

QUESTIONS
• You may wish to discuss this with others before you agree to take part in this study.
• If you have any questions about the research now or during the study contact: Brent Ruby (406) 243-2117 or Steven Gaskill (406) 243-4268.
• If you have any questions regarding your rights as a research subject, you may contact the Chairman, J.A. Rudbach, of the IRB through the Research Office at the University of Montana at 243-6670.

SUBJECT'S STATEMENT OF CONSENT
   I have read the above description of this research study. I have been informed of the risks and benefits involved, and all my questions have been answered to my satisfaction. Furthermore, I have been assured that a member of the research team will also answer any future questions I may have. I voluntarily agree to take part. I understand I will receive a copy of this consent form.

Printed (Typed) Name of Subject

________________________________________________________________________

Subject’s Signature Date

SUBJECT'S STATEMENT OF CONSENT TO BE PHOTOGRAPHED DURING DATA COLLECTION
   I provide my consent to be photographed during periods of the data collection. I realize that these digital images may be used during presentation of the data at regional and national meetings.

________________________________________________________________________

Subject’s Signature Date
Bibliography


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