NOTES

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Quantitation of Absolute ^{2}H Enrichment of Plasma Glucose by ^{2}H NMR Analysis of Its Monoacetone Derivative

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A simple ^{2}H NMR method for quantifying absolute ^{2}H-enrichments in all seven aliphatic positions of glucose following its derivatization to monoacetone glucose is presented. The method is based on the addition of a small quantity of ^{2}H-enriched formate to the NMR sample. When the method was applied to [2-^{2}H]monoacetone glucose samples prepared from [2-^{2}H]glucose standards of known enrichments in the range of 0.2–2.5%, enrichment estimates derived by the NMR method were in good agreement with the real enrichment values of the [2-^{2}H]glucose precursors. The measurement was also applied to monoacetone glucose derived from human plasma glucose samples following administration of ^2H_2O and attainment of isotopic steady state, where glucose H2 and body water enrichment are equivalent. In these studies, the absolute H2 enrichment of plasma glucose estimated by the formate method was in good agreement with the ^{2}H-enrichment of body water measured by an independent method. Magn Reson Med 48: 535–539, 2002. © 2002 Wiley-Liss, Inc.

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Deuterium-enriched water is a safe and practical tracer of hepatic glucose metabolism in animals and humans (1–6). After ingestion, the deuterium rapidly distributes into bulk body water and is incorporated into the aliphatic hydrogens of hepatic glucose-6-phosphate (G6P) derived from both gluconeogenesis and glycogenolysis by various exchange mechanisms (7,8). During fasting, hydrolysis of hepatic G6P accounts for the bulk of plasma glucose, hence, under isotopic steady-state conditions the ^{2}H enrichment pattern of hepatic G6P and plasma glucose are identical. Furthermore, enrichment at position 2 of both plasma glucose and hepatic G6P approaches that of bulk body water due to extensive interchange of G6P hydrogen 2 and water hydrogens via phosphoglucoisomerase (3,9). The ^{2}H-enrichment distribution in hydrogens 2 and 5 and the methylene hydrogen 6 pair of plasma glucose provides information about the relative contributions of glycogen, glycerol, and PEP to total hepatic glucose output (9). Enrichment in these specific positions is quantifiable using GC-MS, but the current derivatization methods are laborious and require considerable technical expertise (9). Recently, relative ^{2}H-enrichments of all seven aliphatic glucose hydrogens were quantified by a much simpler procedure based on ^{2}H NMR analysis of the 1,2-*O*-isopropylidene-D-glucofuranose derivative, also known as monoacetone glucose (MAG) (10). The analysis, while not as sensitive as the GC-MS method, can be performed on ~20 ml of euglycemic whole blood following enrichment of body water to ~0.5%.

Hence, the NMR method is practical for quantifying the contribution of glycogen, glycerol, and PEP to hepatic glucose output in most adults in the clinical setting. However, the ^{2}H NMR measurement does not provide absolute ^{2}H-enrichments of plasma glucose. While the contributions of glycogenolysis and gluconeogenesis to hepatic glucose output can be determined from relative enrichments, absolute ^{2}H-enrichment measurements of the glucose positions could provide additional metabolic information, particularly under nonsteady-state conditions. In this report, we describe a method for obtaining absolute positional ^{2}H-enrichments of plasma glucose by a combination of ^{1}H and ^{2}H NMR spectroscopy of its MAG derivative. Initially, we prepared MAG using acetone enriched with a known quantity of acetone-d_6, with the anticipation that in a ^{2}H NMR spectrum of the product the resolvable ^{2}H signals of the acetone moiety of MAG would provide an absolute intramolecular reference for the other ^{2}H signals. However, this approach proved to be unreliable; thus, we developed an alternative method based on the addition of a small amount of ^{2}H-enriched formate to the sample. We applied the method to human subjects who had ingested tracer amounts of ^2H_2O to compare enrichment of position 2 of plasma glucose with that of body water under isotopic steady-state conditions.

MATERIALS AND METHODS

Human Studies

All subjects were studied under a protocol approved by the University of Coimbra Hospital Ethics Committee. Two healthy subjects and three patients with biopsy-confirmed cirrhosis were studied. The healthy subjects consisted of

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one male, 73 kg, age 25, and one female, 63 kg, age 39. The patient group consisted of two females, ages 22 and 40, weighing 45 and 60 kg, and one male, age 25, weighing 69 kg. Subjects began fasting at 20:00. Subjects ingested a priming dose of ^2H_2O designed to achieve body water enrichment of ~0.5%. To reduce the possibility of vertigo, this was divided into two portions, taken at 01:00 and 06:00 the following day. To ameliorate the unusual taste of highly enriched ^2H_2O, and to further reduce the risk of vertigo, each portion was diluted with ~200 ml of bottled spring water. Body water mass was assumed to be 60% of the total weight for men and 50% for women (6). For the remainder of the study, all subjects ingested 0.5% ^2H_2O in bottled spring water ad libitum. Subjects ingested 250–500 mg phenylbutyric acid packaged in gelatin capsules at 09:40, 10:00, and 10:20, for a total of 0.75–1.5 g. At 11:00, 11:20, 11:40 and 12:00, 20 ml of blood was drawn from a peripheral vein. Urine was also collected between 10:30 and 12:00, at which point the study was concluded.

Sample Processing

Blood was stored at 4°C and centrifuged within 2 hr of being drawn. The plasma supernatant protein was precipitated by centrifugation following addition of 1/10th the plasma volume of 70% perchloric acid. The supernatant was neutralized with KOH and lyophilized following removal of $KClO_4$ by centrifugation. For conversion of plasma glucose to the monoacetone derivative, the lyophilized extracts were treated with 3–20 ml anhydrous acetone enriched to 0.15% with acetone-d_6 to which concentrated sulfuric acid (4% v/v) was added. The solution was quickly cooled to room temperature and monoacetone glucose was prepared using the procedure of Snowden (11). In the final stages of the preparation the solvent was removed by rotary evaporation and monoacetone glucose was extracted from the dried residue with a few milliliters of boiling ethyl acetate or 1–2 ml of 95% acetonitrile / 5% H_2O. After evaporation the residue was dissolved in 0.575 ml of 95% acetonitrile / 5% water containing a few grains of sodium bicarbonate and placed in a 5 mm NMR tube (12). To this, 25 μl of an aqueous potassium formate standard solution of known concentration (0.4003 M) and ^2H-enrichment (4.98%), pH 8.0, was added. MAG standards with ^2H-enrichment levels of 0.21–5.27% in hydrogen 2 were prepared by mixing solutions of unlabeled glucose (19.23 mg/ml) and [2-^2H]glucose enriched to 98% (18.84 mg/ml) followed by lyophilization and derivatization as described. For each standard a total of ~40 mg of glucose was used. In addition to the low-abundance enrichment standards, a nominal 50% enrichment standard was also prepared by combining 529 mg of the unlabeled glucose solution (containing 10.17 mg glucose) and 531 mg of the [2-^2H]glucose solution (containing 10.00 mg 98% [2-^2H]glucose) and its MAG derivative was analyzed by ^1H NMR. From the relative ratios of the MAG hydrogen 1 and hydrogen 2 signals, the ^2H-enrichment of the parent glucose mixture was estimated to be 48.2%, in excellent agreement with the theoretical value of 49.1%.

NMR Spectroscopy

NMR spectra were acquired at 11.75 T with a Varian Unity 500 system equipped with a 5-mm broadband "switchable" probe with z-gradient (Varian, Palo Alto, CA). Deuterium was observed with the "broadband" coil tuned to the ^2H carrier frequency. For this particular probe, detuning the lock coil beforehand resulted in a substantial reduction of the 90° pulse width for ^2H on the broadband coil (~30 μs to 19 μs). Shimming was performed on the ^1H signal using both the Varian automated gradient shimming routine and manual adjustment in response to the ^1H-linewidths of selected resonances. Proton-decoupled ^2H NMR spectra were obtained without lock at 50°C (12,13) using the 19 μs 90° pulse and a 1.2-sec acquisition time. The number of free-induction decays acquired for MAG derived from blood samples ranged from 40,000–60,000 (13.3–20 hr). For the [2-^2H]MAG standards, this number was 1000–5000 (20–100 min). Fully relaxed ^1H NMR spectra were obtained under the same conditions with presaturation of the acetonitrile signal using a pulse width of 45° and delay of 16 sec. Four to 16 free-induction decays were acquired for each spectrum. Deuterium enrichment of urine and plasma water was analyzed by ^2H NMR as previously described (14). A pulse width of 21° and delay of 12.8 sec were used to obtain fully relaxed ^2H NMR signals. Between 40 and 100 free-induction decays were acquired for each spectrum. Analyses were performed in triplicate for each subject and at least two ^2H_2O enrichment standards were run during each NMR session, in addition to a separate calibration curve generated with ^2H_2O enrichment standards ranging from 0.2–1.0%. Stable isotope tracers were obtained from Cambridge Isotopes (Cambridge, MA). T_1 measurements were performed and analyzed using the Varian analysis software. The relative areas of selected peaks in both ^1H and ^2H NMR spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR, Fremont CA). Linear regression analysis of the NMR data was performed with Microsoft Excel.

Calculation of Absolute ^2H-Enrichment of MAG Using the ^2H-Enriched Formate Standard

Given the known ^2H-enrichment of the formate standard (4.98%) and equal amounts of standard and MAG, ^2H-enrichment of MAG hydrogen 2 is simply the formate enrichment multiplied by the ratio of formate and MAG ^2H-signal intensities ($4.98 \times {}^2H\ signal_{MAG2}/{}^2H\ signal_{formate}$). To account for unequal quantities of standard and analyte, this expression is multiplied by the relative amounts of formate and MAG, obtained from the ratio of formate and MAG hydrogen 1 signals in the ^1H NMR spectrum ($^1H\ signal_{formate}/{}^1H\ signal_{MAG1}$). The hydrogen 1 signal was selected over the other ^1H-MAG resonances because it is fully resolved and is furthest away from the solvent signals. Therefore, the percent enrichment of MAG hydrogen 2 was calculated from the following equation:

$$\%\ Enrichment_{MAG2} = 4.98 \times \{{}^2H\ signal_{MAG2}/(1.33 \times {}^2H\ signal_{formate})\} \times (1.052 \times {}^1H\ signal_{formate}/{}^1H\ signal_{MAG1}).$$

A correction factor of 1.052 was applied to the ^1H formate signal to account for the total quantity of formate ($^1H + {}^2H$)

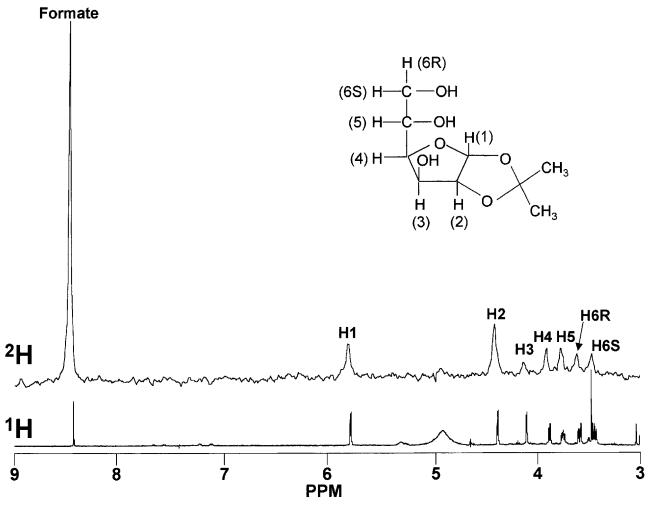

FIG. 1. ^2H and ^1H NMR spectra of a monoacetone glucose sample prepared from plasma glucose obtained from a single blood sample of a healthy patient. The ^2H NMR spectrum represents the sum of 60,000 free-induction decays while the ^1H NMR spectrum represents the sum of four free-induction decays. Also shown is the structure of monoacetone glucose and hydrogen resonance assignments (H1–H6) for the hexose moiety.

present. The ^1H signal of MAG hydrogen 1 was not corrected because the fractional enrichment of ^2H at this site was relatively insignificant (~0.5% or less). In the ^2H NMR measurement, the formate ^2H signal undergoes partial saturation due to its relatively long relaxation time (found to be 2.5 ± 0.7 sec under the experimental conditions). Meanwhile, the deuterium nuclei of MAG with T_1 values, ranging from 180–235 ms, are fully relaxed.

To account for its differential saturation, the ^2H formate signal intensity was multiplied by a correction factor (1.33). The correction factor was obtained by quantifying the change in formate ^2H signal relative to that of MAG hydrogen 2 of selected [2-^2H]MAG standards (1.24, 2.64, and 5.27%) under ambient and fully relaxed conditions. Fully relaxed ^2H NMR spectra were achieved by inserting a pulse delay of 15 sec between acquisitions, giving a total time of 16.2 sec between pulses. Absolute ^2H-enrichments of the other MAG hydrogens were calculated from the ratio of their ^2H-signal relative to that of hydrogen 2 multiplied by the absolute enrichment of hydrogen 2. Where repeated measurements were performed, the data are presented as the mean ± 1 standard deviation.

RESULTS AND DISCUSSION

Derivatization of Glucose With ^2H-Enriched Acetone

This approach is attractive since it requires only the addition of a small quantity of acetone-d_6 to the bulk acetone solvent used in the initial acetonation of plasma glucose. The acetone ^2H-enrichment is preserved in the isopropylidene moiety of MAG, giving rise to a pair of well-resolved ^2H NMR signals at 1.25 and 1.35 ppm with similar T_1 values (276 and 244 ms) to the other MAG ^2H nuclei. The method relies on the assumption that the MAG isopropylidene enrichments reflect those of the parent acetone solvent, i.e., there is no exchange or isotopic discrimination against the ^2H label during the acetonation step. MAG prepared from blood extracts featured well-defined ^1H NMR signals for the hexose moiety of MAG (Fig. 1, bottom). The corresponding ^2H NMR spectra featured narrow, well-resolved signals for the hexose moiety of MAG with natural linewidths of ~2 Hz, (Fig. 1, top). However, the ^2H signals of the isopropylidene moiety at 1.25 and 1.35 ppm had poor lineshapes, with significantly broader linewidths of 5–7 Hz (not shown). As a result, higher uncertainties were associated with quantification of these signal intensities compared to the other MAG resonances. Natural-abundance ^2H NMR spectra of authentic MAG run under similar conditions had narrow and consistent linewidths for all signals, suggesting that the lineshape of the isopropylidene ^2H-resonances observed in our preparations was not an NMR artifact. In the ^1H NMR spectrum, our MAG preparations had several minor signals that were clustered around those of the isopropylidene moiety (not shown). For [2-^2H]glucose standards prepared with enriched acetone, the calculated enrichment in hydrogen 2 of MAG based on the ratio of the isopropylidene and hydrogen 2 ^2H-signal intensities was only ~70% of the theoretical value. Isotopic discrimination or exchange of the deuterium label during acetonation either with the protons of sulfuric acid or with exchangeable protons present in the sample would be expected to deplete enrichment of the MAG isopropylidene hydrogens relative to the theoretical value. This would result in an overestimation of MAG enrichment in the other positions, in direct contradiction to our observations. We hypothesize that ^2H-enriched side products incorporating the acetone-d_6 were formed during the initial acetonation reaction and contributed additional ^1H and ^2H signals in the isopropylidene region of our MAG preparations. This would account for the larger than expected isopropylidene ^2H signals and their nonisochronous appearance. In conclusion, this approach does not appear to be reliable for quantifying absolute ^2H enrichments of plasma glucose using the described derivatization procedure.

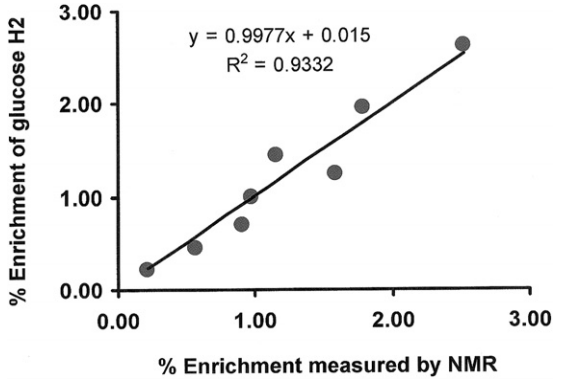

FIG. 2. Relationship between the real enrichment of [2-^2H]glucose preparations vs. the enrichment in hydrogen 2 of their MAG derivatives estimated by the NMR method.

Table 1
Comparison of Body Water Enrichment and Enrichment of Plasma Glucose Hydrogen 2 Measured by the Formate Standard for Each Subject

	^{2}H Enrichment of body water	^{2}H Enrichment of plasma glucose hydrogen 2
Healthy subjects		
1	0.54 ± 0.03	0.49
2	0.40 ± 0.01	0.42
Cirrhotic patients		
1	0.60 ± 0.04	0.59
2	0.46 ± 0.03	0.50
3	0.57 ± 0.04	0.56

Quantification of Absolute ^{1}H Enrichment of MAG Using a ^{1}H-Formate Standard

Under our NMR observing conditions, the formate ^{2}H-enrichment standard generates a narrow, well-resolved, and stable signal in both ^{2}H and ^{1}H NMR spectra with no perturbation of the MAG signals (see Fig. 1). This allowed confident quantification of both ^{1}H and ^{2}H signals of formate and MAG, providing the necessary information for calculating the absolute enrichment of MAG hydrogen 2. For MAG prepared from glucose standards enriched with 0.21–2.64% ^{2}H in hydrogen 2, the NMR measurement of MAG hydrogen 2 enrichment closely matched the known enrichment of the glucose precursors (Fig. 2).

In a different kind of verification experiment, we compared the absolute enrichment of MAG derived from plasma glucose of subjects who had ingested tracer amounts of ^2H_2O with ^{2}H-enrichment of body water. The ingestion protocol was designed to achieve isotopic steady state of body water with equivalent ^{2}H-enrichment in hydrogen 2 of plasma glucose (3). Therefore, our measurement of absolute ^{2}H enrichment of position 2 of MAG should correspond with the ^{2}H-enrichment estimate of body water. As a group, the five subjects had a sizable range of body water enrichments (0.40−0.60%). Nonetheless, these values were closely matched by the MAG H2 enrichment estimates as shown in Table 1. Absolute ^{2}H-enrichment in all positions of MAG is listed in Table 2. Generally, ^{2}H-enrichments in the other positions relative to that of H2 and body water were higher in the patients compared to the healthy subjects, indicating a higher contribution of gluconeogenesis to glucose production for the patients. From the H5/H2 enrichment ratio, the percentage contribution of gluconeogenesis to hepatic glucose output for the three patients was 66, 71, and 73%, compared to values of 46% and 52% for the healthy subjects. The tendency for higher gluconeogenic contributions to hepatic glucose output in the cirrhotic patients compared to healthy controls is consistent with other measurements of gluconeogenesis using ^2H_2O in overnight-fasted cirrhotic subjects (15,16). Furthermore, the H5/H2 enrichment ratio for the healthy subjects in their 16th hour of fasting is in good agreement with recent GC-MS and NMR estimates obtained under similar conditions (3,6,9,15,16).

Our NMR analysis of plasma glucose ^{2}H-enrichment using the monoacetone derivative is based on the pioneering ^{2}H NMR studies of Schleucher et al. (12,13) on glucose derived from plant carbohydrates. We modified their methods to allow the measurement to be performed in conventional NMR tubes and to accommodate the small quantities of MAG generated from plasma glucose samples. Schleucher et al. (13) added a standard amount of C_6D_6 to their MAG samples to obtain absolute ^{2}H-enrichment values. Like formate, C_6D_6 has a narrow and well-resolved signal in the ^{2}H NMR spectrum of the MAG preparation, but requires specialized NMR tubes with vacuum-tight valves (13). In addition, Schleucher et al. were able to obtain several hundred mg of pure crystalline MAG from the plant material, allowing precise amounts to be added to the NMR tube by weighing. Under these conditions the MAG enrichment can be directly determined from the relative ratio of the C_6D_6 and MAG signals in the ^{2}H NMR spectrum. However, this approach is not optimal for routine analysis of plasma glucose, since the amount of MAG recovered is much smaller (~5–10 mg), making crystallization and quantification by weighing impractical. In conclusion, our approach provides a simple and reliable measurement of absolute ^{2}H-enrichment in the aliphatic hydrogens of plasma glucose following administration of ^2H_2O to humans.

Table 2
Absolute ^{2}H Enrichments of Plasma Glucose Hydrogens From Fasted Healthy and Cirrhotic Subjects Following ^2H_2O Ingestion

	^{2}H Enrichment of plasma glucose hydrogens						
	H1	H2	H3	H4	H5	H6*R*	H6*S*
Healthy subjects							
1	0.34	0.51	0.11	0.21	0.27	0.21	0.19
2	0.27	0.42	0.15	0.21	0.19	0.17	0.18
Cirrhotic patients							
1	0.53	0.59	0.34	0.43	0.46	0.35	0.32
2	0.35	0.50	0.24	0.33	0.33	0.25	0.25
3	0.45	0.56	0.29	0.38	0.41	0.28	0.32

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