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Hepatic UDP-Glucose ^{13}C Isotopomers From [U-^{13}C]Glucose: A Simple Analysis by ^{13}C NMR of Urinary Menthol Glucuronide

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Menthol glucuronide was isolated from the urine of a healthy 70-kg female subject following ingestion of 400 mg of peppermint oil and 6 g of 99% [U-^{13}C]glucose. Glucuronide ^{13}C-excess enrichment levels were 4–6% and thus provided high signal-to-noise ratios (SNRs) for confident assignment of ^{13}C-^{13}C spin-coupled multiplet components within each ^{13}C resonance by ^{13}C NMR. The [U-^{13}C]glucuronide isotopomer derived via direct pathway conversion of [U-^{13}C]glucose to [U-^{13}C]UDP-glucose was resolved from [1,2,3-$^{13}C_3$]- and [1,2-$^{13}C_2$]glucuronide isotopomers derived via Cori cycle or indirect pathway metabolism of [U-^{13}C]glucose. In a second study, a group of four overnight-fasted patients (63 ± 10 kg) with severe heart failure were given peppermint oil and infused with [U-^{13}C]glucose for 4 hr (14 mg/kg prime, 0.12 mg/kg/min constant infusion) resulting in a steady-state plasma [U-^{13}C]glucose enrichment of 4.6% ± 0.6%. Menthol glucuronide was harvested and glucuronide ^{13}C-isotopomers were analyzed by ^{13}C NMR. [U-^{13}C]glucuronide enrichment was 0.6% ± 0.1%, and the sum of [1,2,3-$^{13}C_3$] and [1,2-$^{13}C_2$]glucuronide enrichments was 0.9% ± 0.2%. From these data, flux of plasma glucose to hepatic UDPG was estimated to be 15% ± 4% that of endogenous glucose production (EGP), and the Cori cycle accounted for at least 32% ± 10% of GP. Magn Reson Med 56:1121–1125, 2006. © 2006 Wiley-Liss, Inc.

Key words: Cori cycle; glycogen; ^{13}C-isotopomer; Krebs cycle; indirect pathway

The hepatic uridine diphosphate glucose (UDPG) pool is a strategic site for sampling tracers of human hepatic carbohydrate metabolism (1–4). During feeding, there is net hepatic glycogen synthesis from both glucose and gluconeogenic substrates. Carbon fluxes from these sources converge at glucose-6-phosphate (G6P) and then pass via glucose-1-phosphate (G1P) and UDPG pools on the way to glycogen. Consequently, any ^{13}C-enriched precursor of hepatic glycogen is also incorporated into UDPG. During fasting, there is net glucose synthesis from glycogen and gluconeogenic substrates, and carbon fluxes from these sources also converge at G6P. While the bulk of G6P is hydrolyzed to glucose via glucose-6-phosphatase under these conditions, there is a residual flux from G6P through the glucose-1-phosphate (G1P) and UDPG pools. Consequently, any ^{13}C-tracer that enriched the hepatic G6P pool is also incorporated into UDPG. Moreover, the relatively small pool sizes of hepatic sugar and nucleotide diphosphates coupled with extensive G6P-G1P exchange mean that UDPG and G6P enrichments will be equivalent under most ^{13}C-tracer study conditions.

In humans, UDPG can be noninvasively sampled by the administration of drugs such as acetaminophen or diflusinal, which are converted into glucuronides and cleared into urine (4,5). The glucuronide moiety is derived from hepatic UDPG with no modification of the glucose carbon skeleton or dilution of UDPG ^{13}C-enrichment. Glucuronide appearance in urine is rapid, and pharmacological doses of acetaminophen or peppermint oil generate 100–200 μmol of urinary glucuronide per hour (10,11). With this amount of analyte, quantification of ^{13}C-isotopomer populations at 0.1% excess enrichment or less becomes practical by ^{13}C NMR. In the fed state, acetaminophen glucuronide enrichment from infused or ingested ^{13}C-glucose provides information on the pathways of hepatic glycogen synthesis (5–7). In the fasted state, glucuronide ^{13}C-isotopomers from gluconeogenic substrates such as [U-^{13}C]propionate provides a measure of relative gluconeogenic and hepatic Krebs cycle fluxes (8,9). [U-^{13}C]glucose has also been used as a dual tracer of fasting endogenous glucose production (EGP) and glucose recycling (glucose→pyruvate→glucose) via the Cori and glucose-alanine cycles (12–14). Resolution and quantification of glucose triose ^{13}C-isotopomers formed by the recycling pathways have been used to estimate the fractional contribution of the Cori cycle and gluconeogenesis to GP (12–14). However, since the enrichment levels of ^{13}C-isotopomers formed via recycling are typically only 10–20% that of the infused [U-^{13}C]glucose (15), their precise quantification from plasma glucose samples by ^{13}C NMR is often limited by sample size and the relatively large background signals from the parent [U-^{13}C]glucose isotopomer.

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Urinary glucuronide provides five to 10 times more sample mass than a 20-ml blood glucose sample. Furthermore, the contribution of the parent [U-^{13}C]glucose tracer to the glucuronide ^{13}C NMR spectrum is substantially less compared to that of plasma glucose because UDPG synthesis via the direct pathway is suppressed during fasting.

To date, analysis of acetaminophen glucuronide ^{13}C enrichment or isotopomer distributions by either mass spectrometry (MS) or ^{13}C NMR has required rather laborious purification and derivatization methods (4,16). Recently we demonstrated that menthol glucuronide derived from ingestion of peppermint oil can be directly analyzed for ^{2}H enrichment by ^{2}H NMR following a simple isolation procedure (11). In this report we demonstrate that direct ^{13}C NMR spectroscopy of the same menthol glucuronide preparation following administration of [U-^{13}C]glucose and $^{2}H_2O$ tracers provides a wealth of ^{13}C-isotopomer information with no interference from the simultaneous incorporation of ^{2}H into positions 1–5 of glucuronide. Thus, the direct NMR analysis of menthol glucuronide is a practical and effective approach for integrating enrichment information from ^{2}H and ^{13}C tracers of hepatic carbohydrate metabolism.

MATERIALS AND METHODS

Human Studies

Human studies were performed after the subjects provided informed consent, and the study protocols were approved beforehand by the University Hospital of Coimbra Ethics Committee. In the first study, which was designed to generate high levels of glucuronide ^{13}C-isotopomers for ^{13}C signal assignments, a healthy 23-year-old female began to fast at 2130 hr, after she had dinner. She ingested 350 g of 70% $^{2}H_2O$ at 0230 hr the following day, and at 0745 hr she ingested 2 × 200 mg enteric-coated peppermint oil capsules and 6 g of 99% [U-^{13}C]glucose. Urine was collected 2–4 hr after the ingestion of peppermint oil. In a second study, four patients with severe heart failure (NYHA category III/IV, weight = 63 ± 10 kg) began to fast at 2000 hr. They ingested $^{2}H_2O$ to 0.5% body water by drinking two loading doses of 70% $^{2}H_2O$ at 0100 and 0300 hr. At 0600 hr, a 4-hr primed infusion of [U-^{13}C]glucose was started (14 mg/kg prime, 0.12 mg/kg/min constant infusion), and at 0700 hr, 2 × 200 mg enteric-coated peppermint oil capsules were ingested. Urine was collected from 0900 to 1100 hr.

Sample Processing

Urine samples were concentrated 10–20-fold to final volumes of 15–30 ml by rotary evaporation. The concentrated urine was then acidified to pH 1.5 with 6 M HCl. Any precipitate formed was removed by centrifugation, and a saturating amount of NaCl was added to the supernatant. The solution was extracted four to six times with an equal volume of ether, and the ether fractions were pooled. Aliquots containing ~100 μmol of menthol glucuronide were evaporated to dryness. A 10-g Discovery DSC-18 SPE cartridge was conditioned with 17 ml of methanol and 33 ml of acidified water. Acidified water was prepared by mixing 1 ml of 85% H_3PO_4 with 700 ml of distilled water. The menthol glucuronide aliquot was dissolved in 2.5 ml of water, pH 2.5, and applied to the column. The column was then washed with 40 ml of acidified water, followed by two portions of acidified water/methanol (80/20% and 50/50%). Menthol glucuronide was eluted with a third wash of 25/75% acidified water/methanol. To remove phosphoric acid, this fraction was evaporated, dissolved in 2 ml of water (pH 2.5), and applied to a 5-g Discovery DSC-18 SPE cartridge. The column was washed with 20 ml of water, and menthol glucuronide was eluted with 40 ml of 100% methanol. This fraction was evaporated to complete dryness and the residue was dissolved in 0.6 ml of dry acetonitrile-d_3 for ^{13}C NMR analysis.

NMR Spectroscopy

Proton-decoupled ^{13}C NMR spectra were obtained with a Varian Unity 11.75T system equipped with a 5-mm broadband probe. Spectra were obtained with a 60° pulse angle, an acquisition time of 2.5 s, a spectral width of 25 KHz (200 ppm), and a pulse delay of 0.5 s. NMR spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR Inc., Fremont, CA, USA). Menthol glucuronide ^{13}C chemical shifts were referenced to the methyl carbon of acetonitrile at 0.3 ppm.

Metabolic Flux Analyses

To demonstrate the application of ^{13}C-isotopomer analysis of glucuronide for deriving metabolic flux information, we used menthol glucuronide and plasma glucose ^{13}C-isotopomer distributions to estimate the following flux parameters in the group of heart failure patients: Flux from glucose to UDPG was estimated from the fractional enrichment of menthol [U-^{13}C]glucuronide ($E_{glucuronide}$) divided by the enrichment of the plasma [U-^{13}C]glucose precursor pool (E_{plasma}) and multiplied by the endogenous GP rate (EGP), as shown in the following equation:

$$\text{Glucose} \rightarrow \text{UDPG flux } (\mu\text{mol/kg/min}) = \text{EGP} \times (E_{glucuronide})/(E_{plasma}) \quad [1]$$

Cori cycle flux was estimated from the enrichment levels of "first-pass" menthol glucuronide triose isotopomers (E_{triose}) relative to the plasma [U-^{13}C]glucose precursor pool multiplied by EGP. A correction factor of 1.5 was used to account for dilution at the level of the oxaloacetate pool of the hepatic Krebs cycle.[1]

$$\text{Cori cycle flux } (\mu\text{mol/kg/min}) = \text{EGP} \times 1.5 \times (E_{triose})/(E_{plasma}) \quad [2]$$

[1]Enrichment of any [U-^{13}C]anaplerotic tracer that is converted to glucose via gluconeogenesis is diluted at the level of oxaloacetate by the oxidative flux of the Krebs cycle. For the "first-pass" isotopomers, this dilution factor is simply the net anaplerotic flux divided by the sum of anaplerotic plus oxidative (citrate synthase) flux. Assuming that net anaplerosis is 1.5–3.0 times that of oxidative flux in humans (8,9,12), the dilution factor ranges from 1.3–1.7.

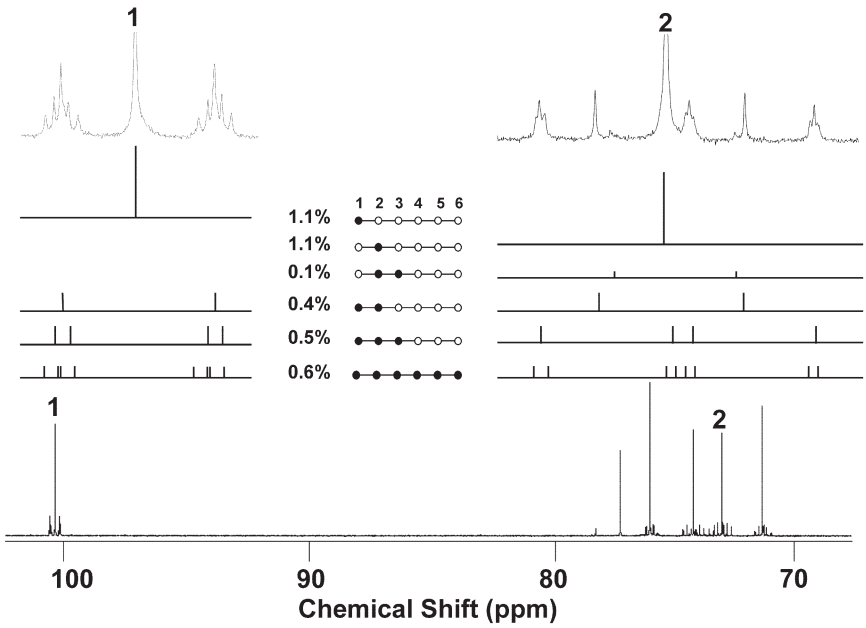

FIG. 1. ^{13}C NMR spectrum of purified menthol glucuronide obtained from urine collected after ingestion of peppermint oil and infusion of [U-^{13}C]glucose. The multiplets of carbons 1 and 2 are shown in expanded form and with a fivefold vertical gain. Assignments of individual ^{13}C-isotopomer components and estimated enrichment levels of selected ^{13}C-hexose and triose isotopomers are also shown.

RESULTS AND DISCUSSION

Menthol glucuronide derived from urinary ether extracts and dissolved in acetonitrile at concentrations of ~0.2 M generated narrow and well-resolved ^{13}C resonances for the six glucuronide carbons. We obtained optimal ^{13}C-signal linewidths of 0.3–0.5 Hz by acquiring the ^{13}C NMR spectrum at 50–70°C. In the single [U-^{13}C]glucose ingestion study designed to provide high ^{13}C-enrichment levels for the assignment of glucuronide ^{13}C-isotopomers, the excess ^{13}C-enrichments were ~6% for glucuronide carbons 1, 2, 5, and 6, and ~4% for carbons 3 and 4. Under these conditions, most of the individual ^{13}C-isotopomer signals intensities were comparable to that of the natural abundance ^{13}C signal, and thus allowed confident identification and assignment.

The ^{13}C-menthol glucuronide signals were also quantifiable during a standard isotope-dilution measurement of fasting EGP with [U-^{13}C]glucose. This substantially enhanced the information content of the study and provided direct insight into fluxes between plasma glucose and hepatic sugar phosphates. Figure 1 shows a ^{13}C NMR spectrum obtained from a heart-failure patient infused with [U-^{13}C]glucose for 4 hr to a steady-state plasma enrichment of 5.8% and administered with peppermint oil. The six glucuronide ^{13}C-resonances consist of singlet signals (mostly derived from natural-abundance ^{13}C) that are flanked by ^{13}C-^{13}C-spin-spin coupled multiplets. The multiplets represent a mixture of glucuronide ^{13}C-isotopomers derived from hepatic metabolism of [U-^{13}C]glucose (Fig. 2). The presence of long-range ^{13}C-^{13}C couplings that span the glucuronide triose moieties (e.g., coupling between carbons 1 and 6) allow the resolution of certain hexose ^{13}C-isotopomer populations. For example, [U-^{13}C]glucuronide generates an eight-line signal in carbon 1 due to a combination of $J_{1,2}$, $J_{1,3}$, and $J_{1,6}$ coupling (see Table 1 and Fig. 1). In the [U-^{13}C]glucose infusion experiment, the probability of forming glucuronide isotopomers containing ^{13}C in positions 1, 3, and 6 by any pathway other than conversion of [U-^{13}C]glucose to [U-^{13}C]UDPG by the so-called "direct pathway" is negligible[2]. Meanwhile, glucuronide ^{13}C-enrichment patterns arising from the Cori cycle (i.e., metabolism of [U-^{13}C]glucose to the level of pyruvate and reincorporation into G6P by gluconeogenesis, as shown in Fig. 2) consist of triose isotopomers, such as [1,2,3-$^{13}C_3$]glucuronide. Such isotopomer populations are well resolved in the ^{13}C NMR spectrum of menthol glucuronide because the $J_{1,2}$ and $J_{2,3}$ coupling constants have different magnitudes, hence [1,2-$^{13}C_2$], [2,3-$^{13}C_2$] and [1,2,3-$^{13}C_3$]glucuronide isotopomers generate distinctive ^{13}C NMR splitting patterns within the carbon 2 resonance. Likewise, different $J_{4,5}$ and $J_{5,6}$ coupling constants mean that the carbon 5 resonance has fully resolved multiplet signals for [4,5-$^{13}C_2$], [5,6-$^{13}C_2$] and [4,5,6-$^{13}C_3$]glucuronide isotopomers. Hence, all possible triose isotopomers that are generated by the metabolism of [U-^{13}C]glucose through the Cori cycle are detectable. ^{13}C-Enriched gluconeogenic substrates, such as [U-^{13}C]propionate, generate the same set of triose ^{13}C-isotopomers, and their relative abundance provides information on anaplerotic and pyruvate recycling fluxes of the hepatic Krebs cycle (8,9,17). In this setting, the menthol glucuronide ^{13}C NMR spectrum will provide well-resolved multiplets in both carbon 2 and 5 resonances, allowing reliable quantification of the triose ^{13}C-isotopomer population. Although it was beyond the scope of this study, the complete resolution of menthol glucuronide ^{1}H signals (11) facilitates the quantification of glucuronide ^{13}C-isotopomer distributions by alternative indirect detection techniques such as J-resolved heteronuclear single quantum coherence (JHSQC) and heteronuclear multiple quantum coherence total correlation spectroscopy (HMQC-TOCSY) (18–20).

[2]In this experimental setting, the probability of synthesizing G6P with two labeled triose moieties via gluconeogenesis or an indirect pathway is very low.

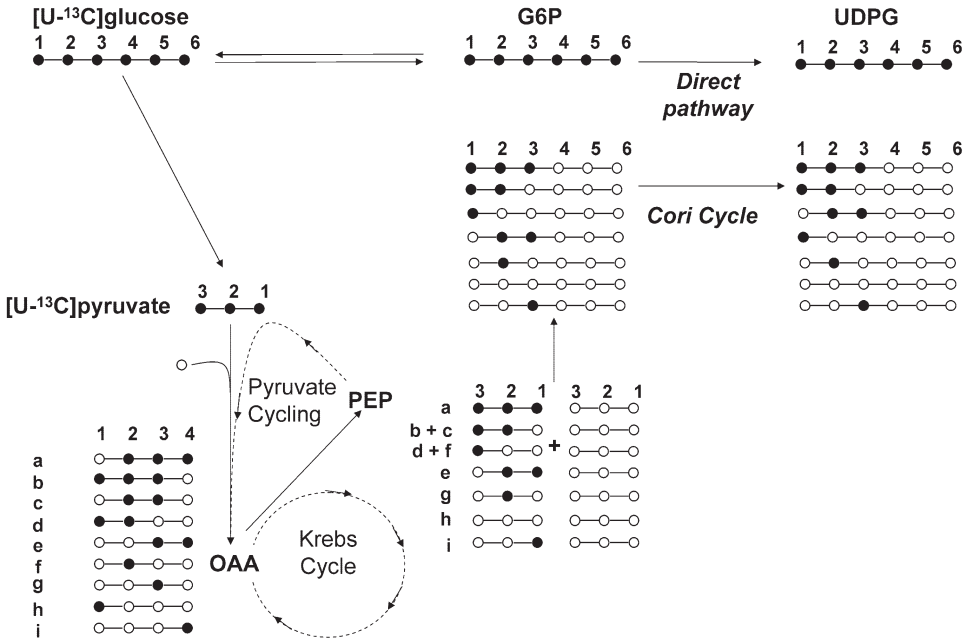

FIG. 2. Formation of oxaloacetate (OAA), phosphoenolpyruvate (PEP) and UDP-glucose (UDPG) ^{13}C-isotopomers during metabolism of [U-^{13}C]glucose via the indirect pathway and hepatic Krebs cycle. For clarity, only triose isotopomers from one half of the UDPG molecule (i.e., carbons 1, 2, and 3) are shown. Isotopomer a is formed by carboxylation of [1,2,3-$^{13}C_3$]pyruvate. OAA-fumarate randomization converts up to 50% of this population to isotopomer **b**. Pyruvate recycling in combination with OAA-fumarate randomization generates isotopomer **c** from both **a** and b (17). Isotopomers **d–i** are derived from ^{13}C-enriched OAA that is metabolized to citrate and regenerated via the oxidative span of the Krebs cycle.

In the four heart-failure patients studied, the enrichment of [U-^{13}C]glucuronide was estimated to be 0.6% ± 0.1%. Plasma [U-^{13}C]glucose enrichment was 4.6% ± 0.6%, and EGP was estimated to be 14.7 ± 1.6 μmol/kg/min (data not shown). From the ratio of [U-^{13}C]glucuronide to [U-^{13}C]glucose enrichment (see Eq. [1]), the flux of glucose to UDPG was estimated at 2.3 ± 1.5 μmol/kg/min corresponding to 15% ± 4% of GP. These data indicate that conversion of plasma glucose to UDPG occurs even during fasting, when the liver is a net exporter of glucose. These estimates are somewhat higher than that reported in a study of overnight-fasted healthy humans by Hellerstein et al. (5), in which the glucose to UDPG flux was estimated to be about 8% of GP.

The enrichment levels of glucuronide triose isotopomers are sensitive to the extent of glucose recycling via the Cori and glucose-alanine cycles. Infused [U-^{13}C]glucose is initially metabolized to [U-^{13}C]pyruvate via glycolysis in peripheral tissues. Subsequently, this product is exported to the liver as [U-^{13}C]lactate or alanine, and incorporated into the gluconeogenic pathway following conversion to [U-^{13}C]pyruvate, carboxylation to OAA via pyruvate carboxylase, and conversion to PEP via PEP-carboxykinase. Due to exchanges at the level of the Krebs cycle, anaplerotic entry of [U-^{13}C]pyruvate will generate eight different PEP ^{13}C-isotopomers, as shown in Fig. 2. Of these, [U-^{13}C]PEP and [2,3-$^{13}C_2$]PEP are derived by "first-pass" metabolism of the [U-^{13}C]pyruvate carbon skeleton through the hepatic OAA pool, and these two isotopomers become [1,2,3-$^{13}C_3$] and [1,2-$^{13}C_2$]glucuronide[3]. Quantification of these isotopomer enrichments relative to that of the plasma [U-^{13}C]glucose precursor provides an estimate of the Cori cycle flux (see Eq. [2]). For the heart-failure patients, the summed enrichment of [1,2,3-$^{13}C_3$] and [1,2-$^{13}C_2$]glucuronide was 0.9% ± 0.1%. From these and the plasma glucose isotopomer data, Cori cycle flux was calculated to be 5.0 ± 2.1 μmol/kg/min corresponding to 32% ± 10% of GP. While this value is high compared to estimates of ~15–20% obtained by NMR and GC-MS analyses of plasma glucose isotopomers following [U-^{13}C]glucose infusion in overnight-fasted healthy subjects (12,14,15), it is similar to the value of 36% reported for healthy subjects fasted for 40 hr (12) and the estimate of 33% for cancer patients with high serum cortisol levels (13). Hence, Cori cycling is elevated when hepatic glycogen stores are depleted by prolonged fasting, or during hypermetabolic conditions as indicated by high cortisol levels. Our finding of comparably high levels of Cori cycle flux in the heart-failure patients is consistent with a growing consensus

[3]An equivalent amount of [4,5,6-$^{13}C_3$] and [5,6-$^{13}C_3$] $^{13}C_2$]glucuronide isotopomers will also be produced.

Table 1
Chemical Shifts and ^{13}C-^{13}C Coupling Constants for the Glucuronide Moiety of Menthol Glucuronide

Glucuronide position	1	2	3	4	5	6
^{13}C Chemical shift (ppm)	100.26	73.49	76.39	71.87	74.61	169.61
^{13}C-^{13}C one-bond coupling constants (Hz)	$^{1}J_{C1-C2}$ 47.6	$^{1}J_{C2-C1}$ 47.6 $^{1}J_{C2-C3}$ 39.5	$^{1}J_{C3-C2}$ 39.5 $^{1}J_{C3-C4}$ 39.3	$^{1}J_{C4-C3}$ 39.3 $^{1}J_{C4-C5}$ 39.6	$^{1}J_{C5-C4}$ 39.6 $^{1}J_{C5-C6}$ 64.5	$^{1}J_{C6-C5}$ 64.5
^{13}C-^{13}C multiple bond coupling constants (Hz)	$^{2}J_{C1-C3}$ 4.7 $^{3}J_{C1-C6}$ 5.5	$^{2}J_{C2-C4}$ 2.4	$^{2}J_{C3-C1}$ 4.7 $^{2}J_{C3-C5}$ 2.8 $^{3}J_{C3-C6}$ 5.1	$^{2}J_{C4-C2}$ 2.4 $^{2}J_{C4-C6}$ 0.8	$^{2}J_{C5-C3}$ 2.8	$^{3}J_{C6-C1}$ 5.5 $^{2}J_{C6-C4}$ 0.8 $^{3}J_{C6-C3}$ 5.1

that severe heart failure is associated with insulin resistance and hypermetabolism (21).

In conclusion, our studies demonstrate that a wealth of ^{13}C-isotopomer information describing human hepatic [U-^{13}C]glucose metabolism can be resolved in a simple manner by direct ^{13}C NMR analysis of urinary menthol glucuronide. This approach can be applied to other ^{13}C-tracers of hepatic gluconeogenesis and glycogen synthesis. The ^{13}C NMR measurement can accommodate and complement isotopic enrichment and metabolic information from ^{2}H-enriched tracers of human glucose metabolism, thereby facilitating the design of highly informative noninvasive tracer assays of hepatic glucose and glycogen metabolism.

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