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Simple Measurement of Gluconeogenesis by Direct ^2H NMR Analysis of Menthol Glucuronide Enrichment from ^2H_2O

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The contribution of gluconeogenesis to fasting glucose production was determined by a simple measurement of urinary menthol glucuronide (MG) ^2H enrichment from ^2H_2O. Following ingestion of ^2H_2O (0.5% body water) during an overnight fast and a pharmacological dose (400 mg) of a commercial peppermint oil preparation the next morning, 364 μmol MG was quantitatively recovered from a 2-h urine collection by ether extraction and a 125 μmol portion was directly analyzed by ^2H NMR. The glucuronide ^2H-signals were fully resolved and their relative intensities matched those of the monoacetone glucose derivative. The pharmacokinetics and yields of urinary MG after ingestion of 400 mg peppermint oil as either gelatin or enteric-coated capsules 1 h before breakfast were quantified in five healthy subjects. Gelatin capsules yielded 197 ± 81 μmol of MG from the initial 2-h urine collection while enteric-coated capsules gave 238 ± 84 μmol MG from the 2- to 4-h urine collection. Magn Reson Med 54:429–434, 2005. © 2005 Wiley-Liss, Inc.

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Deuterated water is widely considered a practical tracer of endogenous glucose production in humans. After ingestion, the deuterium distributes rapidly into bulk body water and is incorporated into numerous metabolites including hepatic glucose-6-phosphate (G6P). During fasting, the ratio of ^2H enrichment in positions 5 and 2 of G6P reflects the relative contribution of glycogenolysis and gluconeogenesis to hepatic G6P synthesis (1). The bulk of fasting plasma glucose is derived from hepatic G6P; therefore, both metabolites share a common ^2H enrichment pattern under isotopic steady-state conditions. On this basis, the contribution of gluconeogenesis to fasting glucose production can be obtained by quantifying ^2H enrichment in positions 5 and 2 of plasma glucose (1–3). This can be achieved by a very sensitive but labor-intensive dehomologation and mass-spectrometry procedure (1,4), or alternatively, by a less sensitive but more convenient ^2H NMR analysis of a monoacetone glucose (MAG) derivative (5–7). MAG is easily prepared from plasma glucose and has fully resolved ^2H NMR signals for all hydrogens attached to the hexose skeleton. Quantification of the 5:2 deuterium enrichment ratio from the relative areas of the ^2H NMR signals of hydrogen 5 and 2 is simple and provides estimates of gluconeogenesis that are consistent with the MS procedure (8). Due to rapid exchange between hepatic G6P and glucose-1-phosphate (G1P), the 5:2 deuterium enrichment ratio of G6P is also preserved in the hexose moieties of G1P, UDP-glucose, and glucuronide. Preliminary studies suggest that the 5:2 deuterium enrichment ratio of urinary paracetamol glucuronide is equal to that of plasma glucose under fasting conditions (9). The high abundance of urinary paracetamol glucuronide allows NMR collection times to be reduced by a factor of 10 or more in comparison to analysis of plasma glucose (9). However, paracetamol glucuronide has poorly dispersed hydrogen NMR signals; hence, it must be derivatized to MAG for ^2H NMR analysis. MAG preparation from urinary glucuronide is considerably more labor intensive than its preparation from plasma glucose (9) and is a significant obstacle for the routine analysis of a large number of urine samples. Therefore, a direct analysis of urinary glucuronide ^2H enrichment with minimal sample processing would be highly desirable for quantifying gluconeogenesis from ^2H_2O. Menthol glucuronide (MG) is a suitable metabolite for such an analysis for the following reasons. First, the chemical shifts of its glucuronide hydrogens are highly dispersed, providing complete resolution of ^2H NMR signals at fields of 11.75 T or higher. Second, menthol glucuronide can be rapidly isolated from urine either as a crude ammonium salt or by simple ether extraction (10). Third, recent studies suggest that 100 μmol[1] or more of urinary menthol glucuronide can be recovered from humans following the consumption of safe quantities of menthol. After ingestion of 100 mg (640 μmol) menthol in gelatin capsules, about 50% was recovered as urinary menthol glucuronide with an elimination half-life of ~1 h

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[1]Given favorable ^2H signal linewidths, this quantity of glucuronide enriched 0.5% ^2H would allow ^2H NMR spectra with high signal to noise ratios (25:1) to be collected in 3 h or less with a conventional 5-mm broadband probe operating at 11.75 T.

(11). No adverse effects were reported following menthol administration and subjects were unable to distinguish between ingestion of menthol and placebo capsules. Similar quantities of menthol can also be safely ingested in the form of peppermint oil, which contains 50–70% menthol and is used as a nonprescription medication to relieve digestive ailments such as irritable bowel syndrome. Dosages of 200–400 mg peppermint oil, administered as gelatin capsules, are considered safe. These capsules are available in both gelatin and enteric-coated formulations. Enteric capsules deliver peppermint oil into the small intestine, thereby eliminating the possibility of esophageal discomfort from gastric reflux of menthol. In this report, we demonstrate that the fractional contribution of gluconeogenesis to hepatic glucose production can be quantified in a very simple manner by direct ^2H NMR spectroscopy of urinary menthol glucuronide following ingestion of ^2H_2O and peppermint oil capsules.

METHODS

Human Studies

Five healthy and nonobese young adults (two females and three males) participated in the studies. All subjects provided informed consent for the peppermint oil and ^2H_2O ingestion studies. One hour before a light breakfast, subjects ingested two gelatin capsules each containing 200 mg peppermint oil suspended in sunflower seed oil (Obbekjaers, Carisan Alps, Herlev, Denmark). Urine was collected every 2 h until 8 h after ingestion. Subjects repeated the same procedure at least 4 weeks later with enteric-coated gelatin peppermint oil capsules. To obtain menthol glucuronide enriched with ^2H in the setting of Landau's ^2H_2O measurement of gluconeogenesis, one of the subjects participated in an additional study. He began fasting at 21:00 and was given ^2H_2O to ~0.5% body water enrichment. This was taken as two loading doses of ~300 mL 35% ^2H_2O taken at 01:00 and 03:00 the following day. At 07:00 the subject ingested two enteric-coated peppermint oil capsules and urine was collected every 2 h until 13:00. No food was ingested during this time.

Sample Processing

Commercial ammonium menthol glucuronide (Sigma-Aldrich) was converted to the acid by passage of 1 mL of 0.1 M solution through ~1 mL of Dowex-50-H^+-200–400 mesh ion-exchange resin in a Pasteur pipette followed by 5 mL water. The water was evaporated and the menthol glucuronic acid was dissolved in 0.6 mL of dry acetonitrile-d_3. Urine samples were concentrated about 10- to 20-fold to final volumes of 15–30 mL by rotary evaporation. The pH was adjusted to 7.0 with NaOH and an aliquot was removed for quantification of menthol glucuronide by ^1H NMR. The concentrated urine was then acidified to pH 1.5 with concentrated HCl. Any precipitate formed was removed by centrifugation and a saturating amount of NaCl was added to the supernatant. The solution was extracted 4–6 times with an equal volume of ether until the ether fraction was devoid of color. The ether fractions were pooled and weighed and an aliquot representing ~1% of the total was reserved for quantification of menthol glucuronide by ^1H NMR. For quantification of ^2H enrichment by ^2H NMR, the ether extract was thoroughly dried by rotary evaporation and the residue dissolved in 1–2 mL dry acetonitrile. The sample was heated to 75°C and incubated for 5–6 h. Any insoluble material that was formed by this treatment was removed by centrifugation. An aliquot of the clear supernatant containing 125 μmol of the glucuronide was evaporated to dryness and resuspended in 0.6 mL dry acetonitrile for ^2H NMR analysis.

Derivatization of Menthol Glucuronide to Monoacetone Glucose

The menthol glucuronide ether extract was dissolved in 5 mL of water and 2 mL of Dowex-50-H^+ ion exchange resin was added. The mixture was boiled for 8 h resulting in the complete hydrolysis of menthol glucuronide to menthol and glucuronic acid. The ion-exchange resin was separated from the mixture by filtration and washed with 5 mL water. The wash was combined with the filtrate and evaporated to dryness at 40°C. The residue was further dried over molecular sieves for 48 h resulting in the conversion of glucuronic acid to lactone. The lactone was converted to its monoacetone derivative by stirring for 24 h with 5 mL of anhydrous acetone and 0.3 g of anhydrous Dowex-50-H^+-200-mesh ion-exchange resin. The ion-exchange resin was then removed by filtration, the pH was adjusted to 4–5 with 0.5 M Na_2CO_3, and the solution was evaporated to dryness at room temperature. Monoacetone glucuronic lactone was extracted from the residue with 1–2 mL acetonitrile. On evaporation of acetonitrile, the monoacetone glucuronic lactone was reduced in aqueous solution with sodium borohydride to MAG (12). Excess borohydride was destroyed by passage of the solution through a 2- to 3-mL column of Dowex-50-H^+ ion exchange resin. Under these conditions, acid-catalyzed hydrolysis of MAG to glucose was minimal. The elutate was evaporated to dryness and then dissolved in 10 mL methanol and evaporated again. Methanol addition and evaporation was repeated three more times to completely remove boric acid as the volatile methyl borate. The residue was dissolved in a small quantity of water and the pH was adjusted to 12 by the addition of 2 M Na_2CO_3. The basic solution was evaporated and MAG was extracted with two 1- to 2-mL portions of boiling ethyl acetate. For NMR spectroscopy, the ethyl acetate was evaporated and the residue resuspended in 90% acetonitrile/10% water (5). Yields of MAG from menthol glucuronide were about 20%.

NMR Spectroscopy

All ^1H and ^2H NMR spectra were obtained with a Varian Unity 11.75-T system equipped with a 5-mm broadband probe. Menthol glucuronide ^1H chemical shifts were assigned from ^1H COSY spectra of commercial menthol glucuronide and urine ether extracts. For quantification of urinary menthol glucuronide, an aliquot of the concentrated urine was assayed for menthol glucuronide by addition of 25 μL of a 0.4 M sodium formate solution and 0.3 mL 99% ^2H_2O. The sample volume was then adjusted to 0.6 mL with water and fully relaxed ^1H NMR spectra were obtained at 25°C with presaturation of the water signal. The upfield methyl ^1H signals of the menthol moiety of

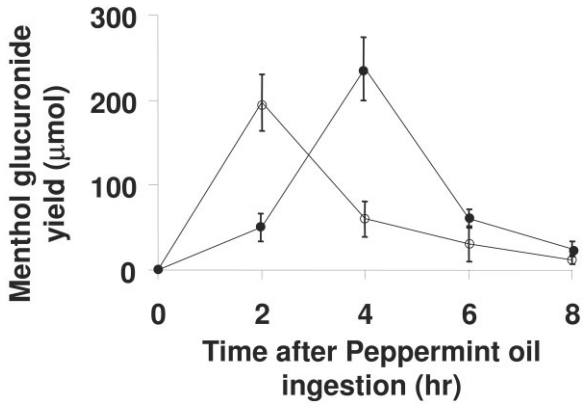

FIG. 1. Quantity of menthol glucuronide from urine samples taken every 2 h after ingestion of plain gelatin capsules (open circles) and enteric-coated gelatin capsules (closed circles). Each datum represents the mean quantity from five individuals and its error bar represents the SD.

menthol glucuronide were quantified relative to that of formate signal. Menthol glucuronide from aliquots of ether extracts was quantified in the same manner after evaporation of ether, resuspension of the residue in 0.3 mL water, and adjustment of the solution pH to 7.0 with 1 M NaOH. Proton-decoupled ^{2}H NMR spectra were obtained in the unlocked mode at a temperature of 75°C with a 90° pulse, a 1.5-sec acquisition time, and no interpulse delay. Spectra of monoacetone glucose derived from the menthol glucuronide were obtained with the same equipment and acquisition parameters at a temperature of 50°C (6,13). Absolute ^{2}H enrichment of the monoacetone glucose hydrogens was quantified as described (13) except that dimethylformamide-d_7 was used as a standard instead of formate-*d*. Thus, the ^{2}H intensities of the three dimethylformamide signals were compared with those of monoacetone glucose after correction for partial saturation effects. Deconvolution and quantification of ^{1}H and ^{2}H NMR signals was performed with the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR, Inc., Fremont, CA, USA).

RESULTS

Recovery of Urinary Menthol Glucuronide from Peppermint Oil

Ingestion of 400 mg peppermint oil in plain gelatin capsules before a light breakfast meal resulted in the rapid appearance of urinary menthol glucuronide with the greatest amount being recovered from the first 2 h of urine collection (Fig. 1). With enteric-coated capsules, the appearance of urinary menthol glucuronide was systematically delayed, with the highest quantity being recovered from urine collected 2–4 h after peppermint oil ingestion. This delay presumably reflects the time taken for the peppermint oil capsule to reach the small intestine before its dissolution. The peak yields of urinary menthol glucuronide were similar for both capsules, with ~200 μmol being harvested from a 2-h urine collection. Between 70 and 90% of urinary MG was recovered by simple ether extraction (data not shown) resulting in 140–180 μmol of glucuronide being available for direct ^{2}H NMR analysis. In the single fasted study where ^2H_2O was ingested, enteric-coated peppermint oil was given and a somewhat higher yield of MG (364 μmol) was recovered from the 2- to 4-h urine collection. This may reflect a more efficient absorption of menthol in the absence of ingested food.

Positional ^{2}H-Enrichment Analysis of Menthol Glucuronide by ^{2}H NMR

The five hydrogen NMR signals from the glucuronide moiety of menthol glucuronide are highly dispersed (Fig. 2).

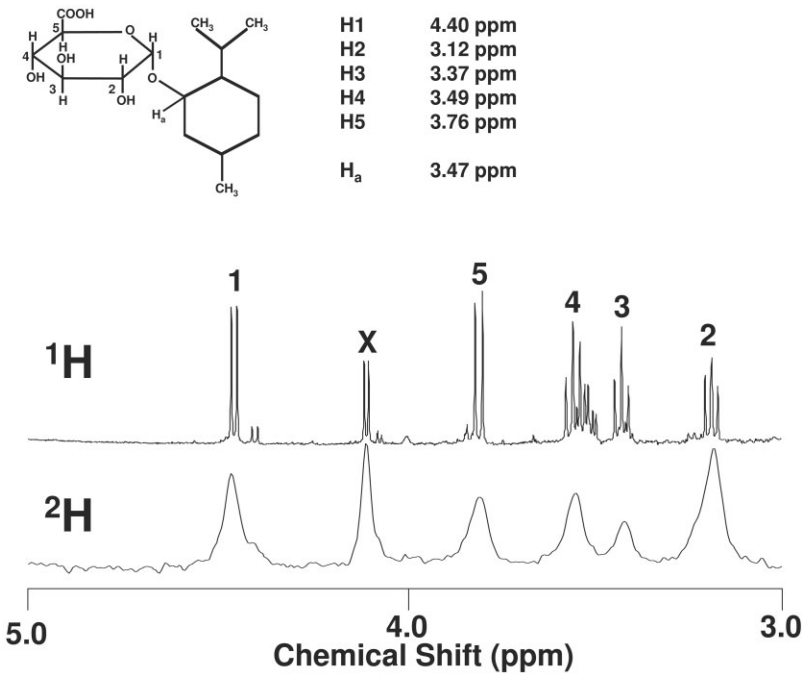

FIG. 2. ^{1}H and ^{2}H NMR spectra of a portion of an ether extract obtained from urine collected after ingestion of peppermint oil and ^2H_2O. The sample contained 125 μmol menthol glucuronide. The ^{1}H spectrum represents the sum of 2 acquisitions while the ^{2}H spectrum represents the sum of 5000 acquisitions for a total collection time of 2.1 h. Spectra were acquired consecutively at 75°C without lock. The numbers above the signals represent the position of the resonating hydrogen or deuterium in the parent hepatic glucose-6-phosphate molecule. Also shown is the menthol glucuronide structure and assignments of its hydrogen NMR signals in the 3.0- to 5.0-ppm region.

	MAG	MG
D1	74	70
D2	100	100
D3	29	28
D4	52	53
D5	52	56

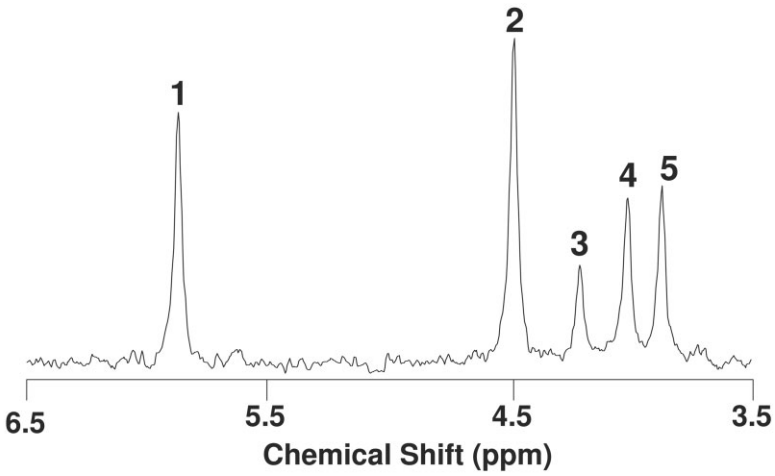

FIG. 3. ^{2}H NMR spectrum of monoacetone glucose (65 μmol) derived from menthol glucuronide. The spectrum represents the sum of 20000 acquisitions for a total collection time of 8.3 h. The numbers above each signal represent the position of the resonating deuterium in the parent hepatic glucose-6-phosphate molecule. Relative ^{2}H-enrichments for positions 1–5 (D1–D5) of monoacetone glucose (MAG) and menthol glucuronide (MG) are shown above the spectrum. In both cases, ^{2}H-enrichment of hydrogen 2 was arbitrarily set to 100.

Apart from the single hydrogen adjacent to the ether linkage (designated H_a in Fig. 2), the signals of the menthol moiety resonate well upfield from those of the glucuronide. Also shown in Fig. 2 are ^{1}H and ^{2}H NMR spectra of menthol glucuronide obtained by ether extraction of urine following ^2H_2O and peppermint oil ingestion. The ^{1}H NMR spectrum shows well-resolved glucuronide resonances with relatively little contamination from other ether-soluble components of urine. All glucuronide proton resonances have a corresponding ^{2}H signal in the ^{2}H NMR spectrum consistent with incorporation of deuterium from ^2H_2O into all positions of hepatic G6P. A single additional ^{2}H signal from an unknown metabolite is also present but does not interfere with any of the glucuronide resonances. The glucuronide ^{2}H NMR signals had linewidths of 3–4 Hz: significantly broader than the typical ^{2}H signals of the MAG derivative (~2 Hz). Nevertheless, the resonances are sufficiently resolved to allow confident quantification of their relative areas at 11.75 T. The T_1 values of the glucuronide ^{2}H nuclei were not quantified but were assumed to be not greater than those of MAG (i.e., 250 ms or less).

The MG extract was then derivatized to MAG and analyzed by ^{1}H/^{2}H NMR in order to confirm the ^{2}H relative enrichment distributions derived from MG and to determine the absolute ^{2}H enrichment values by addition of a deuterated standard (13). The relative ^{2}H enrichment values obtained from the ^{2}H NMR spectrum of the glucuronide were consistent with those derived from a ^{2}H NMR analysis of its MAG derivative (Fig. 3). Absolute ^{2}H enrichment values of the glucuronide moiety, as estimated from the analysis of MAG and DMF signals, ranged from 0.11 to 0.39%. These values are consistent with recent measurements of fasting plasma glucose ^{2}H enrichments following ingestion of ^2H_2O to ~0.5% body water (6). The gluconeogenic contribution to hepatic glucose output as calculated from the glucuronide 5:2 ^{2}H-enrichment ratio was estimated to be 56%, consistent with recent MS and NMR measurements of gluconeogenesis by the ^2H_2O method in overnight-fasted healthy subjects (2,3,5,6).

DISCUSSION

We demonstrated a novel noninvasive method for quantifying gluconeogenesis from ^2H_2O by ^{2}H NMR analysis of urinary menthol glucuronide. Sample processing is simple and can be performed rapidly if needed. High-quality spectra with a conventional 500-MHz NMR system and broadband probe can be obtained with short collection times. Menthol glucuronic acid is highly soluble in acetonitrile and other organic solvents and is therefore compatible with automated NMR systems and microprobes. Moreover, the simple ^{2}H NMR spectrum of MG obtained after ^2H_2O ingestion study could potentially be processed by an automated Bayesian analysis of the free-induction decay, as was recently demonstrated with the MAG derivative of plasma glucose (14). Integration of these methods could allow the study of much larger subject populations than was hitherto possible. However, there are some subject groups that cannot safely tolerate the dosages of peppermint oil used in this study. Peppermint oil ingestion is contraindicated for patients with severe liver disease, gallbladder inflammation, or obstruction of the bile ducts. Its effects on pregnant women or infants are not known. Administration of peppermint oil capsules to small children is not recommended since there is a risk of the capsule being chewed open, resulting in irritation of the mouth and esophagus. In a study of older children, peppermint oil in enteric-coated capsules was given three times a day to children 8–17 years old and weighing at least 30 kg (15).

Each dose consisted of 187–374 mg of peppermint oil (i.e., 47–94% of the amount used in our study). No adverse effects from the peppermint oil were reported.

Quantification of the gluconeogenic fraction of endogenous glucose production from glucuronide relies on the assumption that both plasma glucose and glucuronide originate from a common G6P pool and therefore have equal ^2H enrichment distributions from ^2H_2O. To date, this has not been thoroughly examined in humans. In a small group of healthy overnight-fasted subjects given ^2H_2O, [U-^{13}C]propionate, and acetaminophen, glucuronide and glucose had consistent ^2H and ^{13}C enrichment distributions (9). However, other carbon tracer studies revealed significant differences between glucose and glucuronide labeling, suggesting that the two metabolites could not have originated from the same G6P precursor pool (16).

Nonequivalent glucose and glucuronide ^2H enrichment distributions could potentially arise from the compartmentalization of glucose metabolism at both hepatic and systemic levels. In the hepatic lobule, the periportal region is considered the principal site of glucose production while the pericentral region is the principal location for UDP-glucose synthesis (17). Steep arteriovenous concentration gradients exist for some gluconeogenic substrates, such as glycerol, but not for others, such as lactate. This implies that the contribution of glycerol to gluconeogenesis is relatively higher in the arterial periportal region compared to pericentral and perivenous zones. In accord with this, Ekberg et al. showed that gluconeogenic incorporation of [2-^{14}C]glycerol relative to [1-^{14}C]lactate was higher in plasma glucose compared to glucuronide (16). However, the extent to which changes in utilization of individual precursor substrates modifies the total gluconeogenic contribution to G6P production is not known. The contribution of glycerol to postabsorptive plasma glucose production is minor (18); hence, a reduced concentration of glycerol is unlikely to substantially change the fraction of G6P derived from gluconeogenesis in pericentral compared to that of periportal hepatocytes.

The liver accounts for ~95% of glucuronide secretion (19); hence, the glucuronide ^2H enrichment distribution reflects that of hepatic G6P. Fasting plasma glucose has long been assumed to be largely derived from liver, but recent studies have demonstrated a significant capacity for nonhepatic glucose synthesis in humans. Gluconeogenic carbon tracers were incorporated into plasma glucose during the anhepatic phase of liver transplantation (20). A functional G6P phosphohydrolase protein was identified in several extrahepatic tissues including skeletal muscle (21). Although this enzyme is less active than hepatic G6P-ase, it is considered sufficiently abundant to make a significant contribution to whole-body glucose production. To the extent that the sources of G6P in these tissues differ from those in liver, glucose derived from extrahepatic sources will have a different ^2H enrichment distribution compared to that of hepatic glucose.[2] If there were no exchanges between plasma glucose and intracellular glucose of various tissues, the plasma glucose ^2H enrichment distribution would represent a weighted average of hepatic and extrahepatic sources of glucose production while the glucuronide ^2H enrichment distribution would be specific for the liver. In reality, plasma and hepatic glucose pools are exchanged by futile cycling between hepatic G6P and glucose via glucokinase and G6P-ase (22). Presumably, extrahepatic tissues that produce glucose undergo a similar exchange process via a futile cycle catalyzed by hexokinase and G6P phosphohydrolase. By homogenizing the various G6P pools, these exchanges minimize any differences between glucose and glucuronide ^2H-enrichment distributions that might be attributable to extrahepatic glucose production.

In conclusion, we describe a simple method of quantifying the glucuronide ^2H enrichment distribution based on the ingestion of peppermint oil and a simple recovery of urinary menthol glucuronide. Where applicable, this approach should considerably simplify the measurement of gluconeogenesis by Landau's ^2H_2O method.

[2]For example, gluconeogenesis is not active in muscle hence glucose production would be largely derived from glycogenolysis. Glucose from this source would have a lower 5:2 ^2H-enrichment ratio compared to hepatic glucose.

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