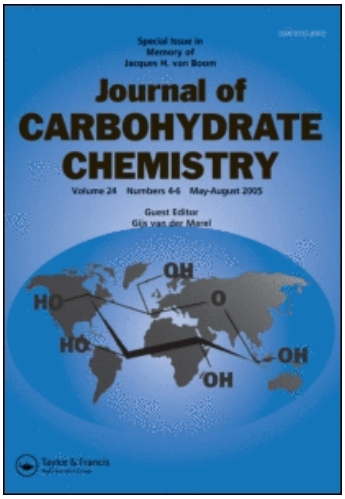

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John G. Jones[a]; Cristina Barosa[a]; Filipe Gomes[b]; Ana Carina Mendes[b]; Teresa C. Delgado[a]; Luisa Diogo[c]; Paula Garcia[c]; Margarida Bastos[d]; Luisa Barros[d]; Ana Fagulha[d]; Carla Baptista[d]; Manuela Carvalheiro[d]; M. Madalena Caldeira[b]

[a] NMR Research Unit, Department of Biochemistry and Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal [b] Department of Chemistry, University of Coimbra, Coimbra, Portugal [c] Pediatrics Hospital of Coimbra, Coimbra, Portugal [d] Department of Endocrinology, University Hospital of Coimbra, Coimbra, Portugal

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NMR Derivatives for Quantification of ^{2}H and ^{13}C-Enrichment of Human Glucuronide from Metabolic Tracers

John G. Jones and Cristina Barosa

NMR Research Unit, Department of Biochemistry and Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal

Filipe Gomes and Ana Carina Mendes

Department of Chemistry, University of Coimbra, Coimbra, Portugal

Teresa C. Delgado

NMR Research Unit, Department of Biochemistry and Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal

Luisa Diogo and Paula Garcia

Pediatrics Hospital of Coimbra, Coimbra, Portugal

Margarida Bastos, Luisa Barros, Ana Fagulha, Carla Baptista, Manuela Carvalheiro

Department of Endocrinology, University Hospital of Coimbra, Coimbra, Portugal

M. Madalena Caldeira

Department of Chemistry, University of Coimbra, Coimbra, Portugal

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Address correspondence to John G. Jones, NMR Research Unit, Department of Biochemistry and Center for Neurosciences and Cell Biology, Faculty of Sciences and Technology, University of Coimbra, Coimbra 3001-401, Portugal. E-mail: jones@cnc.cj.uc.pt

Quantification of ^{2}H and ^{13}C enrichment distributions in human urinary glucuronide following ingestion of $^{2}H_2O$ and ^{13}C gluconeogenic tracers was achieved by NMR spectroscopy of the 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone and 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone derivatives. The derivatization process is simple and can be applied to any glucuronide species. The derivatives are highly soluble in acetonitrile and generate well-resolved and narrow ^{2}H and ^{13}C NMR signals. The 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone derivative provided resolution of the six glucuronide ^{13}C signals and numerous ^{13}C isotopomer populations through one- and two-bond ^{13}C-^{13}C-coupling, while the 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone derivative provided complete resolution of the ^{2}H NMR signals for the five glucuronide hydrogens. The isopropylidene methyl signals were also resolved and provided an internal ^{2}H enrichment standard following the acetonation of glucuronolactone with deuterated acetone.

Keywords Isotopomers, Gluconeogenesis, Intermediary metabolism, Glycogen, Liver

INTRODUCTION

Carbon-13 and deuterium tracers are now widely used in human studies of carbohydrate metabolism.[1–6] While mass spectroscopy (MS) analysis is more established and widespread due to lower instrumentation cost and better sensitivity, NMR spectroscopy is becoming increasingly available due to the proliferation of medium field (400–600 MHz ^{1}H) spectrometers that have vastly improved sensitivity and performance over their predecessors. NMR is particularly effective for resolving complex enrichment patterns from mixtures of ^{13}C and ^{2}H tracers,[4,7,8] thereby providing the basis for the design of highly informative tracer studies for measurement of metabolic fluxes. Labeling information for each isotope is represented by NMR signals whose resolution and quantification rests on two main characteristics. First is the chemical shift and coupling constant, which determine the position and multiplicity of the signal in the NMR spectrum. Second is the NMR sample environment, which can have a significant influence on the signal linewidth. For monosaccharides such as glucose and glucuronide, both characteristics can be substantially optimized by relatively simple derivatization methods coupled to the use of a low-viscosity organic solvent such as acetonitrile.[9,10]

Beyond the extraction of metabolites and separation from salts and proteins, there is typically no further processing of a biological sample for NMR analysis of isotope enrichment. With ^{13}C NMR, the large chemical shift dispersion means that well-resolved ^{13}C signals from many ^{13}C-labeled metabolites are available for quantification. This is useful for providing a general overview of the ^{13}C enrichment or isotopomer distributions, and in principle, all metabolite isotopomer or enrichment information can be integrated into a metabolic flux analysis. Usually, however, metabolic flux estimates are obtained from ^{13}C enrichment or isotopomer distributions of a few abundant

metabolites such as lactate, glutamate, or glucose. Moreover, in our experience, concentrated biological preparations are problematic since they contain high salt concentrations. This increases the heating effect from broadband proton decoupling, resulting in sample temperature instabilities and a degradation of signal quality. Also, pulse widths are altered resulting in poor reproducibility of signal intensities, particularly with indirect-detection measurements of ^{13}C-enrichment and isotopomer distributions. For these reasons, metabolite derivatives that are relatively salt-free and soluble in organic solvents are highly advantageous for both ^{2}H and ^{13}C NMR observation.

In human studies, NMR isotopic measurements of carbohydrate metabolism are typically focused on plasma glucose or glucuronide recovered from urine.[11–13] A diverse range of xenobiotic and pharmacological compounds, including menthol and paracetamol, are eliminated from the body by hepatic glucuronidation and clearance of the glucuronide into urine. In all cases, the glucuronide moiety is derived from hepatic uridine diphosphate glucose (UDPG); hence, the provision of an appropriate xenobiotic agent and subsequent recovery of urinary glucuronide permits a noninvasive "chemical biopsy" of UDPG (Fig. 1). Paracetamol is the most widely used glucuronide

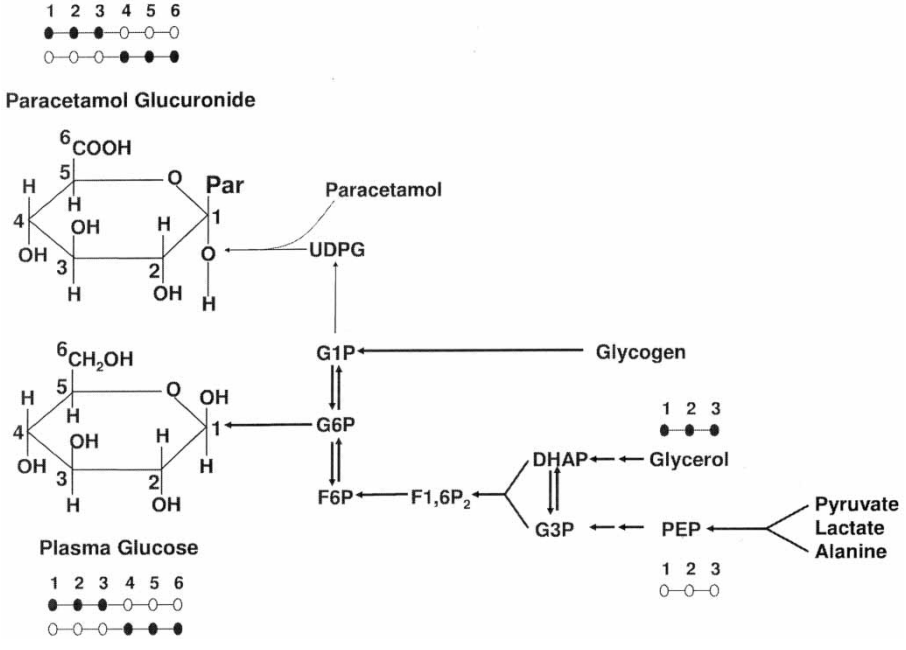

Figure 1: Major biochemical pathways of plasma glucose and paracetamol glucuronide synthesis during fasting. Also shown pictorially are the hexose ^{13}C isotopomers that are synthesized by the incorporation of (U-^{13}C)glycerol into the gluconeogenic pathway directly via triose phosphates. The shaded circles represent ^{13}C atoms while the open circles represent ^{12}C atoms.

probe since it is one of the most commonly used analgesics and a pharmacological dose generates abundant quantities of urinary paracetamol glucuronide. Following provision of a ^{13}C-enriched gluconeogenic precursor such as [U-^{13}C]propionate or [2-^{13}C]glycerol, the ^{13}C isotopomer distribution of glucuronide provides information on gluconeogenic fluxes.[3,13] The advent of $^{2}H_2O$ as a complementary tracer of gluconeogenesis[5,14] focused our interest on improving the hydrogen chemical shift dispersion of paracetamol glucuronide so that ^{2}H-enrichment distribution of the glucuronide hydrogens could also be quantified by ^{2}H NMR.[7] This was achieved by conversion of paracetamol glucuronide to 1,2-*O*-isopropylidene glucofuranose, also known as monoacetone glucose (MAG), but the procedure is quite laborious and MAG is not very soluble in pure organic solvents. In this report, we describe alternative isopropylidene derivatives prepared from both paracetamol and menthol glucuronides. Compared to MAG, they are much simpler to synthesize and are highly soluble in organic solvents. They were found to be effective for resolving complex ^{13}C- and ^{2}H-enrichment patterns from the incorporation of ^{13}C and ^{2}H tracers into human glucuronide.

EXPERIMENTAL

Human Studies

Human studies were performed according to study protocols approved by the Ethics Committees of the University and Pediatrics Hospitals of Coimbra after informed consent was obtained from the subjects. A 19-year-old healthy female volunteer ingested 1,000 mg paracetamol along with a cornflour meal (1 g cornflour/kg body weight). Three hours later, she ingested 1 g [U-^{13}C]glycerol along with a second cornflour meal. Urine was collected between 2 and 4 h after the [U-^{13}C]glycerol ingestion.

In a second study, a 23-year-old female began fasting at 20:00 following a standard dinner. At 01:00 and 03:00, she ingested 0.25 g/kg body water of $^{2}H_2O$. For the remainder of the study, she drank water containing 0.5% $^{2}H_2O$ to maintain body water enrichment. At 07:45, she ingested 2×200 mg enteric-coated peppermint oil capsules and 6 g 99% [U-^{13}C]glucose. Urine was collected from between 2 and 4 h following the ingestion of peppermint oil and menthol glucuronide was isolated from urine by bulk ether extraction of acidified urine. The ether was evaporated and the menthol glucuronide was dissolved in 50 mL water and the pH adjusted to 4.5 to 5.0 with 1 M NaOH. The glucuronide was hydrolyzed by β-glucuronidase and converted to monoacetone derivatives using the same method as for paracetamol glucuronide (see below).

Derivatization of Urinary Paracetamol Glucuronide

Urine was concentrated to 10% of its original volume and this portion was mixed with nine volumes of 100% ethanol. The precipitate was centrifuged, the supernatant was evaporated to ~10 mL, and the pH was adjusted to 8.0 with 1 M NaOH. The supernatant was applied to 18 cm × 1 cm diameter Dowex-1X8-200-acetate column. The column was washed with 35 mL water, the glucuronide eluted with 35 mL 10 M acetic acid, and the acetic acid fraction evaporated to dryness at 40 to 50°C. The residue was resuspended in 50 mL water and the pH was adjusted to 4.5 to 5.0.

For the derivatization of paracetamol glucuronide, 2,000 units of β-glucuronidase (*H. Pomatia*, Sigma Chemical Company) were added and the solution was incubated at 45°C for 48 h. The solution was then passed through 10 mL Dowex-50X8-200 H^+ ion-exchange resin and evaporated to complete dryness at 40°C, resulting in the conversion of free glucuronic acid to glucuronolactone. The lactone was converted to its monoacetone derivative by stirring for 24 h with 5 mL of anhydrous acetone and 0.1 mL concentrated H_2SO_4. The yellow solution was quenched with 5 mL water, the pH adjusted to 4 to 5 with 0.5 M Na_2CO_3 and the solution evaporated to dryness at rt. The 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone product, also known as monoacetone glucuronolactone (MAGL), was extracted from the residue with 1 to 2 mL acetonitrile. On evaporation of acetonitrile, MAGL was resuspended in 0.6 mL anhydrous non-deuterated HPLC-grade acetonitrile (Aldrich, Milwaukee, WI) for NMR analysis. MAGL yields from the starting material ranged from 30% to 50%. The 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone derivative (MAGLA) was prepared from MAGL by adding 121 μL of pyridine and 242 μL of acetic anhydride per 100 μL of lactone and incubating for 18 h at 4°C. The mixture was passed through 1 mL Dowex-50-H^+ and concentrated to dryness. The residue was dissolved in dry acetonitrile for NMR analysis. Yields of MAGLA from MAGL were 70% to 90% giving an overall yield of 20% to 45% from the starting material. Yields were determined by ^{1}H NMR. The product was dissolved in ~2 mL acetonitrile, are the solution was thoroughly mixed and then weighed. A 50- to 100-μL aliquot was added to a 5-mm NMR tube containing 400 μL 99% deuterated acetonitrile and 50 μL of a 0.1 M dimethylformamide (DMF) solution in nondeuterated acetonitrile. All analyte and DMF solutions were weighed on addition to the NMR tube. Fully relaxed ^{1}H NMR spectra were acquired and the area of the MAGL or MAGLA hydrogen 1 signal relative to the DMF methyl signals was quantified to give the quantity of product.

Synthesis of 2% Deuterated Acetone

For preparation of 2% randomly deuterated acetone, 2 mol of $^{2}H_2O$, 18 mol of H_2O, and 80 mol of acetone were combined, the pH of the solution was

adjusted to 11 to 12 with 10 N NaOH, and the solution was stirred for 24 h. The solution was then neutralized to pH 7.0 with 6 N HCl and distilled. The 56 to 58°C fraction was collected and dried over 40 g anhydrous $CaSO_4$ for 24 h, then distilled a second time and stored under anhydrous conditions. This procedure yielded about 40 to 50 mol of acetone whose methyl groups were enriched to 2% with ^2H. To minimize the possibility of ^2H-label dilution via exchange of acetone hydrogens with those of the sulphuric acid catalyst, sulphuric acid enriched to 2% with D_2SO_4 was used for all acetonation reactions. Enrichment of the acetone methyl groups after incorporation into MAG was checked by acetonation of 99% [U-d_7]glucose and integration of the methyl and hexose ^2H NMR signals.

NMR Spectroscopy

All spectra were acquired at 11.75 T and with a Varian Unity 500 system equipped with a 5-mm broadband "switchable" probe with z-gradient (Varian, Palo Alto, CA). Proton-decoupled ^2H NMR spectra were obtained without field-frequency lock at 25°C with a 90° pulse angle, a sweep width of 767 Hz (10 ppm), an acquisition time of 1.6 seconds (1,216 data points) and no pulse delay. Number of acquisitions ranged from 5,000 to 30,000 (2.2–13.3 h). Partially relaxed and proton-decoupled ^{13}C NMR spectra were obtained with following addition of 100 μL CD_3CN to provide a lock signal. Spectra were obtained with a 60° pulse angle, an acquisition time of 2.5 seconds (125,696 data points), a sweep width of 25 KHz (200 ppm), and a pulse delay of 0.5 second. Number of acquisitions ranged from 8,000 to 20,000 (6.7–16.7 h). The WALTZ-16 decoupling sequence was used for proton decoupling of both ^2H and ^{13}C. ^2H free induction decays (FIDs) were multiplied by an exponential function corresponding to 0.5 to 1.0 Hz line-broadening before Fourier transform while ^{13}C FIDs were processed with 0.1 to 0.5 Hz line broadening. All NMR spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR Inc., Fremont, CA).

For human ingestion studies, sterile and pyrogen-free [U-^{13}C]glycerol and [U-^{13}C]glucose was obtained from Cambridge Isotopes, Cambridge, MA. Sterile and pyrogen-free 70% ^2H_2O was obtained from Cambridge Isotopes and Eurisotop, Gif-sur-Yvette, France.

RESULTS AND DISCUSSION

^{13}C NMR Analysis of Glucuronide ^{13}C-isotopomers

Figure 2 shows a ^{13}C NMR spectrum of urinary paracetamol glucuronide obtained after ingestion of [U-^{13}C]glycerol following partial purification by

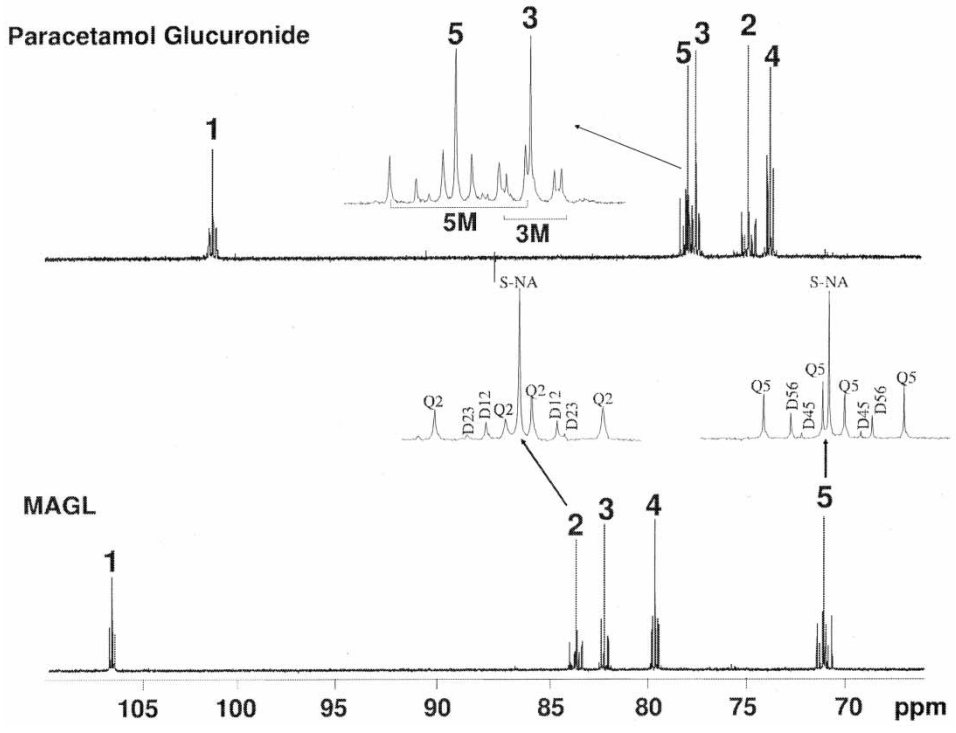

Figure 2: ^{13}C NMR spectrum of Paracetamol glucuronide obtained from urine of a subject who had ingested 2 g (U-^{13}C)glycerol 2–4 h beforehand. The spectrum represents the sum of 18,500 FIDs and took 15.4 h to acquire. The region containing the carbon 3 and 5 resonances and the span of the ^{13}C-^{13}C spin-coupled multiplets for each carbon (3M, 5M) is shown in expanded form. Below is the ^{13}C NMR spectrum of the 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (MAGL) derivative (9,000 FIDs., 6.25 h). The carbon 2 and 5 resonances are shown in expanded view and their ^{13}C-isotopomer components are labeled as follows: S-NA = natural abundance singlet; Q2 = quartet signal from (1,2,3-$^{13}C_3$)glucuronide; D12 = doublet signal from (1,2-$^{13}C_2$)glucuronide; D23 = doublet signal from (2,3-$^{13}C_2$)glucuronide; Q5 = quartet signal from (4,5,6-$^{13}C_3$)glucuronide; D56 = doublet signal from (5,6-$^{13}C_2$)glucuronide; D45 = doublet signal from (4,5-$^{13}C_2$)glucuronide.

anion-exchange chromatography. The signals consist of a natural-abundance singlet flanked by multiplets, which arise as a result of splitting from ^{13}C-^{13}C-coupling interactions (see Table 1). Such signals are expected from this experiment given that the incorporation of [U-^{13}C]glycerol into the triose moieties of glucose-6-phosphate (G6P) and UDPG via gluconeogenesis will generate multiply enriched species (i.e., [1,2,3-$^{13}C_3$] and [4,5,6-$^{13}C_3$]hexose isotopomers). In the ^{13}C NMR spectrum of paracetamol glucuronide, the signals of carbon 3 and 5 are not resolved and the multiplet components cannot be precisely quantified. Moreover, the natural signal linewidths of ~1.5 Hz are insufficiently narrow to resolve two-bond ^{13}C-^{13}C-couplings, such as that between carbon 1 and 3. Conversion to MAGL results in the complete resolution of all

Table 1: Chemical shifts and ^{13}C-^{13}C coupling constants for 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (MAGL) and 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (MAGLA).

		Glucuronide positions						Methyl groups		
		1	**2**	**3**	**4**	**5**	**6**	**A1**	**A2**	**Ac**
PG	^{1}H chemical shift (ppm)	5.10	3.58	3.58	3.58	4.08				
	^{13}C chemical shift (ppm)	101.0	73.4	76.1	72.3	76.5	175.6			
MAGL	^{1}H chemical shift (ppm)	5.96	4.80	4.79	4.87	4.53		1.32	1.49	
	^{13}C chemical shift (ppm)	106.9	83.0	81.5	78.9	70.3	174.2	25.9	26.3	
	$J_{C\text{-}C}$ (Hz)	$^{1}J_{C1\text{-}C2}$ 33.7 $^{2}J_{C1-C3}$ 1.2 $^{3}J_{C1-C6}$ 2.7	$^{1}J_{C2-C1}$ 34.3 $^{1}J_{C2-C3}$ 45.9 $^{3}J_{C2-C5}$ 0.8	$^{1}J_{C3-C2}$ 45.9 $^{1}J_{C3-C4}$ 30.6 $^{2}J_{C3-C1}$ 1.2	$^{1}J_{C4-C3}$ 30.6 $^{1}J_{C4-C5}$ 41.5 $^{2}J_{C4-C6}$ 1.9	$^{1}J_{C5-C4}$ 41.5 $^{1}J_{C5-C6}$56.5 $^{3}J_{C2-C5}$0.8	$^{1}J_{C6-C5}$ 56.5 $^{2}J_{C6-C4}$ 1.9 $^{3}J_{C1-C6}$ 2.7			
MAGLA	^{1}H chemical shift (ppm)	5.96	4.81	4.90	4.99	5.62		1.29	1.44	2.06
	^{13}C chemical shift (ppm)	106.5	82.0	82.1	76.9	69.6	170.0	25.2	25.6	19.8
	J_{C-C} (Hz)	$^{1}J_{C1-C2}$ 34.3 $^{4}J_{C1-C5}$ 3.1	$^{1}J_{C2-C1}$ 34.3 $^{1}J_{C2-C3}$ 32.7	$^{1}J_{C3-C2}$ 32.7 $^{1}J_{C3-C4}$ 30.7 $^{2}J_{C3-C5}$ 1.9	$^{1}J_{C4-C3}$ 30.7 $^{1}J_{C4-C5}$ 42.2 $^{2}J_{C4-C6}$ 1.9	$^{1}J_{C5-C4}$ 42.2 $^{1}J_{C5-C6}$60.6 $^{2}J_{C5-C3}$1.9 $^{3}J_{C5-C2}$1.1 $^{4}J_{C5-C1}$3.1	$^{1}J_{C6-C5}$ 60.6 $^{2}J_{C6-C4}$ 1.9 $^{3}J_{C6-C3}$ 2.6			

six glucuronide ^{13}C signals. Moreover, MAGL is highly soluble in acetonitrile, a low-viscosity solvent that ensures fast molecular tumbling and short correlation times, which result in narrow NMR signals of 0.3 to 0.5 Hz linewidths. Under these conditions, the ^{13}C isotopomer signals are fully resolved, resulting in a better description of the ^{13}C-isotopomer populations (see insets of Fig. 2). This is illustrated by the carbon 5 signal, where signals representing [5,6-$^{13}C_2$-]glucuronide and [4,5,6-$^{13}C_3$]glucuronide are resolved from each other after conversion of paracetamol glucuronide to MAGL. The presence of the [5,6-$^{13}C_2$-]glucuronide isotopomer indicates that a significant portion of the [U-^{13}C]glycerol passed through the hepatic Krebs cycle prior to incorporation into glucose-6-P and glucuronide. The proportions of the corresponding isotopomers for the 1,2,3-triose moiety of glucuronide (i.e., [1,2-$^{13}C_2$] and [1,2,3-$^{13}C_3$]glucuronide), revealed by the carbon 2 multiplet, match those of the [4,5,6-$^{13}C_3$]triose moiety (see Table 2). This signifies that carbon skeleton rearrangements via the transaldolase/transketolase reactions of the pentose phosphate pathway was not significant and therefore could not have contributed significantly to the observed ^{13}C-isotopomer distribution. Measurements of glycerol gluconeogenesis by ^{13}C-glycerol tracers to date[15] have not accounted for its incorporation via the Krebs cycle and therefore likely underestimate its real contribution to gluconeogenesis. MAGL that was derived from the $^{2}H_2O$/[U-^{13}C]glucose ingestion study had significant levels of the [U-^{13}C]glucuronide isotopomer (data not shown). Its ^{13}C NMR multiplet pattern featured ^{13}C-^{13}C coupling between carbons 3 and 4 that were not observed in the [U-^{13}C]glycerol experiment. In addition, there were long-range ^{13}C-^{13}C-couplings between carbons 2 and 5 and carbons 1 and 6 (see Table 1). This abundance of ^{13}C-^{13}C-coupling interactions allowed the resolution of complex mixtures of glucuronide hexose as well as triose ^{13}C isotopomers by ^{13}C NMR.

In this type of experiment, ^{13}C excess enrichment from the tracer can be quantified using the natural abundance singlet signal, representing 1.1% ^{13}C enrichment, as an internal standard (see Table 2). This requires the assumption that metabolism of the tracer will not generate significant levels of singly ^{13}C-enriched hexose isotopomers.[8] In cases where this assumption is not valid (e.g., metabolism of [2-^{13}C]glycerol), the ^{13}C excess enrichment can be obtained from the ^{1}H NMR spectrum, where signals from hydrogens attached to ^{13}C are split by ^{13}C-^{1}H coupling whereas those attached to ^{12}C are not. The background contributions from natural abundance ^{13}C can also be accounted for in this analysis.[16] Alternatively, the natural abundance ^{13}C signals of the MAGL methyl groups can be used as internal standards, but these need to be corrected via calibration curves to account for nuclear Overhauser effects and relaxation differences between the methyl and hexose signals.[17] The 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone derivative (MAGLA) was less suitable for the observation of ^{13}C isotopomers because of the proximity of the carbon 2 and carbon 3 signals (see

Table 2: Total ^{13}C-enrichment, ^{13}C-excess enrichment, and triose isotopomer populations for urinary paracetamol glucuronide enriched with (U-^{13}C)glycerol as measured from the ^{13}C NMR spectrum of the 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (MAGL) derivative.

Glucuronide carbon	**1**	**2**	**3**	**4**	**5**	**6**
^{13}C enrichment (%)	3.1	3.1	2.3	2.8	3.2	3.0
^{13}C excess enrichment (%)	2.0	2.0	1.2	1.7	2.1	1.9
Glucuronide isotopomer	(1,2,3-$^{13}C_3$)	(1,2-$^{13}C_2$)	(2,3-$^{13}C_2$)	(4,5-$^{13}C_2$)	(5,6-$^{13}C_2$)	(4,5,6-$^{13}C_3$)
Abundance (%)	1.5	0.3	0.1	0.1	0.4	1.6

Table 1). There was extensive overlap of ^{13}C-^{13}C spin coupled multiplets of the two resonances, and in addition, the coupling of carbons 2 and 3 generated a complex non–first-order splitting pattern because the chemical shift difference between the two ^{13}C signals ($\sim$10 Hz) was smaller than the coupling constant ($\sim$33 Hz).

^{2}H NMR Analysis of Glucuronide ^{2}H-enrichment from $^{2}H_2O$

Following enrichment of body water with $^{2}H_2O$, all positions of G6P are enriched with ^{2}H as a result of exchange between bulk water and the hydrogens of gluconeogenic precursors and sugar phosphate intermediates. There is also extensive exchange between G6P and glucose-1-phosphate (G1P) catalyzed by phosphoglucomutase; hence, the enrichment pattern of G6P is preserved in the hexose moieties of UDPG and glucuronide (Fig. 1). The enrichment of G6P hydrogen 2 approaches that of body water because of extensive G6P and fructose-6-phosphate (F6P) exchange, catalyzed by G6P-isomerase.[5] However, other hydrogens, including hydrogen 5, have less ^{2}H enrichment as a result of glycogenolysis. This is because G6P molecules derived from glycogenolysis experience only G1P-G6P-F6P exchange, and hence they become enriched in positions 1 and 2 but not in any other site. Enrichment of hydrogen 5 relative to that of hydrogen 2 or body water provides information on the relative rates of G6P synthesis from hepatic glycogenolysis and gluconeogenesis.[5,14] This parameter is easily derived by measuring the intensities of the ^{2}H NMR signals of hydrogen 5 and hydrogen 2, provided that they are well resolved.[4,7,18] As seen in Table 1, MAGL has well resolved hydrogen 1, 4, and 5 signals, but hydrogens 2 and 3 have coinciding ^{2}H NMR resonances, thereby preventing a direct measurement of the hydrogen 5 and hydrogen 2 relative enrichments. Complete resolution of the glucuronide ^{2}H signals was achieved by converting MAGL to MAGLA, as shown in Figure 3. The acetylation reaction provided good yields and was more convenient than the borohydride reduction of MAGL to MAG. In the ^{2}H NMR spectrum the relative ^{2}H-enrichment distribution of all five hydrogens derived from glucuronide is directly proportional to the relative areas of their ^{2}H NMR signals (see expanded view). Hydrogen 2 has the largest signal and therefore the highest relative enrichment, consistent with extensive exchange of this site with body water via G6P-isomerase. The other sites, notably 3, 4, and 5, have less ^{2}H enrichment, reflecting dilution by G6P derived from hepatic glycogenolysis.[4,7,18] In the MAGLA spectrum shown in Figure 3, the ratio of the glucuronide 5 to glucuronide 2 signal was 0.7, indicating that 30% of G6P production had been derived from glycogenolysis and 70% from gluconeogenesis.

Quantification of absolute positional ^{2}H enrichments by ^{2}H NMR requires the addition of an internal deuterated standard such as deuterated benzene, formate, or dimethylformamide to the sample.[10,17,19–21] For the relatively

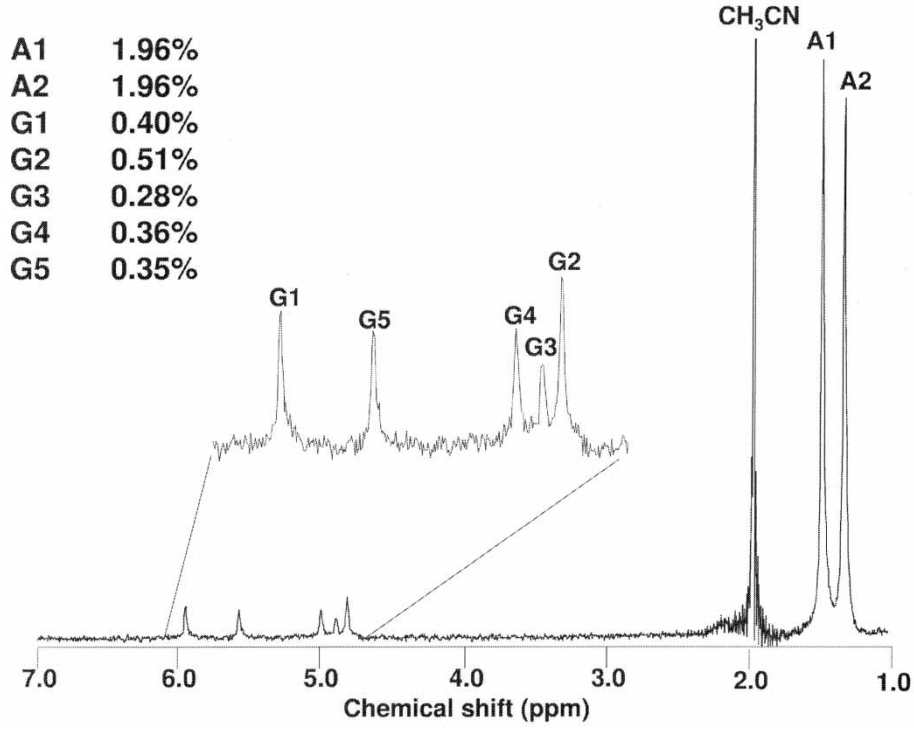

Figure 3: ^{2}H NMR spectrum of 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (MAGLA) prepared from the glucuronide of a healthy subject who ingested $^{2}H_2O$ to ~0.5% body water. The glucuronide was derivatized using acetone enriched to 2% with ^{2}H (see methods). The signals derived from the glucuronide moiety (G1–G5) are shown in expanded view. Also shown are the two methyl signals derived from the deuterated acetone (A1, A2). Absolute ^{2}H enrichment of the glucuronide hydrogens, as estimated from the relative areas of the methyl and glucuronide signals, are also shown.

small sample amounts derived from metabolites, this measurement is cumbersome since the relative amounts of analyte and standard in the NMR sample need to be determined by ^{1}H NMR[19] or other quantitative analytical method. The methyl groups derived from the acetonation reaction provide an opportunity for incorporating an intramolecular ^{2}H-enrichment standard. Moreover, their T_1 values are comparable with those of the other sites (~300 ms), thereby allowing fully relaxed ^{2}H spectra to be acquired with reasonably short recycle times. We synthesized 2% randomly deuterated acetone by base-catalyzed exchange of acetone and D_2O and prepared a MAG derivative with 99% [U-d_7]glucose. In the ^{2}H NMR spectrum, the theoretical ratio of ~99:6 for the signals derived from glucose versus the methyl signals derived from acetone was observed (data not shown). In the glucuronide derivative, the methyl signals derived from the acetonation step (A1, A2 in Fig. 3) were well resolved, providing an internal ^{2}H-enrichment standard for

quantifying absolute glucuronide ^{2}H enrichments (Fig. 3). The absolute ^{2}H-enrichment level of glucuronide position 2 (G2) of ~0.5% is consistent with the extensive exchange between this hydrogen and that of body water (also enriched to ~0.5%).

The precision of ^{2}H-enrichment and ^{13}C-isotopomer measurements ultimately depends on the signal-to-noise ratio (SNR) of the resonance being measured. Following processing of the FIDs. with exponential multiplication, the SNR of the Fourier-transformed spectrum defines the minimum level of uncertainty associated with quantification of signal areas.[22] With this criterion, the minimum uncertainty associated with the quantification of the ^{2}H MAGLA signals in Figure 3 ranges from 5% for the hydrogen 2 signal (SNR = 18.3) to 10% for the hydrogen 3 resonance (SNR = 9.0). For the ^{13}C multiplets of carbon 5 shown in Figure 2, the most intense (Q5) had SNRs of 40 to 50, corresponding to a 2.5% minimum level of uncertainty, while the least intense (D45) component had SNRs of 6 to 8, corresponding to 13% to 17% uncertainty.

In summary, glucuronide agents provide a strategic yet accessible sampling site for hepatic carbohydrate tracers in humans. We have shown that NMR spectroscopy of simple glucuronide derivatives is a practical and effective method for resolution and quantification of complex enrichment patterns from mixtures of ^{13}C and ^{2}H tracers. The derivatization procedure can be performed on any glucuronide species. In the case of paracetamol, the most widely used glucuronidation agent, ingestion of a pharmacological amount (500–1000 mg) generates 200–800 μmol of paracetamol glucuronide over a 2-h urine collection. From this quantity, between 70 and 300 μmol of MAGL or MAGLA can be prepared in 150 to 500 μL of acetontitrile solvent (~0.5–2.0 M analyte concentration). Even at the upper concentration limit, the NMR spectra have narrow signals (0.5 Hz linewidths for ^{13}C, 1.5 Hz linewidths for ^{2}H), allowing effective quantification of signal intensities. Consequently, under these optimal observation conditions, the low glucuronide ^{2}H- or ^{13}C-excess enrichments that are typical for human tracer studies can be reliably quantified by standard NMR instrumentation using reasonably short collection times.

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