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Noninvasive evaluation of liver metabolism by ^{2}H and ^{13}C NMR isotopomer analysis of human urine

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Abstract

Mammalian liver disposes of acetaminophen and other ingested xenobiotics by forming soluble glucuronides that are subsequently removed via renal filtration. When given in combination with the stable isotopes ^{2}H and ^{13}C, the glucuronide of acetaminophen isolated from urine provides a convenient "chemical biopsy" for evaluating intermediary metabolism in the liver. Here, we describe isolation and purification of urinary acetaminophen glucuronide and its conversion to monoacetone glucose (MAG). Subsequent ^{2}H and ^{13}C NMR analysis of MAG from normal volunteers after ingestion of ^{2}H$_2$O and [U-^{13}C$_3$]propionate allowed a noninvasive profiling of hepatic gluconeogenic pathways. The method should find use in metabolic studies of infants and other populations where blood sampling is either limited or problematic.
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Assessment of fluxes through the central metabolic pathways of human hepatic glucose production is essential to the understanding of pathogenesis and the consequences of therapy in several major diseases, including type II diabetes and cirrhosis. In humans, these fluxes are typically evaluated by administration of precursors enriched with ^{2}H or ^{13}C in conjunction with mass spectroscopy or NMR analysis of plasma glucose. For example, Landau and co-workers have shown, following the oral administration of ^{2}H$_2$O, that the ^{2}H-enrichment distribution of glucose provides a direct measure of the contributions of glycogenolysis and gluconeogenesis to glucose production [1,2]. This technique has attracted considerable interest because of the rapid increase in incidence of type II diabetes in human populations. Similarly, oral or intravenous administration of a

gluconeogenic ^{13}C tracer, such as [U-^{13}C$_3$]propionate, can provide metabolic flux information at the level of the hepatic citric acid cycle, the major source of gluconeogenic carbons and a key regulatory site for gluconeogenesis [3–7]. We recently demonstrated that NMR analysis of plasma glucose enriched with a combination of ^{2}H and ^{13}C tracers provides a comprehensive picture of glycogenolysis, gluconeogenesis, and citric acid cycle flux in humans [6].

In the aforementioned human studies, 20–30 ml of blood was required for high-quality NMR spectra [4,6]. This quantity of blood may prove prohibitive for the evaluation of metabolism in neonates, children, elderly, or patients with infectious blood such as in HIV or hepatitis. An alternative method for sampling the plasma glucose pool is to perform a "chemical biopsy" using acetaminophen [8,9]. After a single therapeutic dose (10–12 mg/kg), acetaminophen is metabolized in the liver by microsomal enzymes to acetaminophen-glucuronide (60%), acetaminophen-sulfate (35%),

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acetaminophen-cysteine (3%), and a residual percentage oxidized by cytochrome P450 enzymes [10]. Acetaminophen glucuronide (AG)[1] is formed by condensation of acetaminophen with UDP-glucose (UDPG). The glucose moiety of UDPG is derived from hepatic glucose 1-phosphate, which is in rapid exchange with glucose 6-phosphate, the direct precursor of intrahepatic glucose.

After purification, 300–900 μmol of AG can be sampled from urine of a normal adult, about 4–12 times the amount of glucose recovered from a 30-ml blood sample. In a previous report, we demonstrated that the ^{13}C-isotopomer data could be obtained by ^{13}C NMR after conversion of urinary AG to glucuronic acid [4]. However, proton chemical-shift dispersion of this product is inadequate for resolving and quantifying ^{2}H enrichment in all positions by ^{2}H NMR. Given that the 1,2-diisopropylidene glucofuranose (MAG) derivative of glucose offers excellent chemical-shift dispersion for both ^{2}H [6] and ^{13}C NMR analyses, we focused on the chemical conversion of AG to MAG. Although a few literature accounts of conversion of AG to glucose exist [8,11,12], there are no reports of successful conversion of AG to MAG. The method presented here provides sufficient MAG for NMR studies, is spectroscopically clean, and gives good chemical-shift dispersion of all ^{1}H, ^{2}H, and ^{13}C signals.

Methods

Materials

[U-[13]C$_3$]propionate (99%) and [2]H$_2$O (70%) were purchased from Cambridge Isotopes (Andover, MA). Acetaminophen (1000 mg tablets) was purchased from a local pharmacy. DSC-18 solid-phase extraction (SPE) gel was obtained from Supelco (St. Louis, MO). Other common chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Tracer and acetaminophen administration

All samples were obtained from normal adult volunteers who provided informed consent prior to the study. The protocol was approved by the institutional review board (IRB) at the Dallas Veterans Affairs Medical Center prior to patient enrollment. Five individuals were studied. Following an early evening meal, subjects fasted overnight prior to the study. They reported to the Clinical Research Unit between 0700 and 0800. Over the first hour, the tracers were given orally in three divided doses. All subjects received a total of 5 g/kg body water deuterium oxide and 10 mg/kg body weight of [U-^{13}C]propionate. With the first dose of tracers, the patients also received 1000 mg of acetaminophen. Then 20–30 ml of blood was drawn at 2, 3, and 4 h following ingestion of the final tracer dose. At 4 h, the subjects also voided their bladders with all urine being retained for NMR analysis.

Acetaminophen-glucuronide isolation

The amount of 100–300 ml of urine was collected from patients and immediately sterilized by treatment with 50 ml of acetone for 2 h [13]. The pH of the urine was then adjusted to pH 4.5 with 70% perchloric acid and the volume was reduced by ~50% using rotary evaporation at 50 °C and 30 mm Hg. This solution was diluted to its original volume with water and treated with 10,000 U of urease. The pH of the sample was maintained near 7 by adding drops of 70% perchloric acid every 30–60 min as necessary. Finally, the pH was reduced to 4.5, the solution was filtered, lyophilized to dryness, and refrigerated until further use.

Acidic water (pH 2.5) was prepared by adding 1 ml of concentrated phosphoric acid to 700 ml of deionized water. Thirty grams of SPE gel was loaded into a 60 ml syringe and treated with 50 ml of 100% methanol followed by 100 ml of acidic water. The freeze-dried, urea-free, urine sample was dissolved in a minimum amount of water (typically 3–10 ml) and the pH was adjusted to 2–2.5. The sample was loaded onto the column and washed with 120 ml of acidic water, 120 ml of 10% methanol in acidic water, and finally 120 ml of 100% methanol. The 10% methanol washing was collected, the pH was adjusted to 7, and the solvent was removed under vacuum to give a light-colored solid. This represented 70–88% of total urinary glucuronide as measured by HPLC.

Conversion of AG to MAG

Acetaminophen-glucuronide methyl ester. The purified urinary AG was dissolved in 50 ml of dry methanol, 300 μl of BF$_3$ was added dropwise, and the mixture was stirred overnight at room temperature. Typically, HPLC analyses indicated 90–98% conversion to the ester. Whenever HPLC showed lower amounts of ester formation, those samples were once again freeze-dried and the reaction was repeated.

Acetaminophen-glucoside. Following conversion to the ester, 1200 mg of NaBH$_4$ was added (in 60 mg portions over 3 h) to the same reaction flask. The reduction, as indicated by either HPLC or ^{1}H NMR, was typically 89–98% complete. If glucoside formation was less than ~90%, additional 60 mg portions of NaBH$_4$ were added

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[1] *Abbreviations used:* AG, acetaminophen glucuronide; UDPG, UDP-glucose; MAG, 1,2-diisopropylidene glucofuranose; SPE, solid-phase extraction; HPLC, high-performance liquid chromatography; LFP, liver flux profile.

until conversion reached the desired level. The pH of the solution was adjusted to 2 with concentrated HCl and the solvent was removed under vacuum to give a brown residue. The residue was washed 3×2 ml of methanol and any remaining salt was removed by filtration. The washes were combined and evaporated.

Glucose. The glucoside was dissolved in 25 ml of water, and the pH was adjusted to 7.4 and stirred overnight with 10,000 U of β-glucosidase. This solution was lyophilized and redissolved in a minimal amount of water and applied to a 5-g SPE column, prepared as described above. The glucose was eluted with 40 ml of water leaving colored impurities behind. The colorless wash was lyophilized to a white solid consisting of 40–60 mg of glucose (by enzymatic assay) and ~200 mg of salt.

1,2-Isopropylidene glucofuranose. MAG was synthesized using slight modifications of a previously reported procedure [2]. The glucose/salt solid was pulverized and suspended in 10 ml of dry acetone. Four hundred microliters of concentrated H_2SO_4 was added and the mix was stirred for 8 h at room temperature. Ten milliliters of water was added and the pH was adjusted to 2.0 with 5 M Na_2CO_3. This solution was stirred for another 24 h or until completion as indicated by ^1H NMR. The pH was adjusted to 8.0 with 5 M Na_2CO_3 and the solvent removed under vacuum. The resulting solid was washed with 5×3 ml of boiling ethyl acetate. The washings were combined and the solvent was removed under vacuum leaving a yellow residue. A 5-g SPE cartridge was prepared by washing with 20 ml of methanol followed by 60 ml of water. The residue was dissolved in 0.5 ml of water and applied to the SPE cartridge. The cartridge was washed with one fraction of 20 ml of water (to remove impurities), 40 ml of 10% methanol (to remove pure MAG), and 20 ml of methanol (to completely clean the column).

Hydrogen exchange during synthesis

The synthetic steps described above were also run using a commercial sample of AG (Sigma, St. Louis, MO) in the presence of various deuterated solvents and reactants to test for hydrogen exchange during the synthesis of MAG. The following five conditions were examined: (1) esterification and reduction reactions were performed in 50% [U-$^2H_4$]methanol, (2) the reduction reaction was performed with 98% sodium borodeuteride, (3) hydrolysis of AG by β-glucosidase was performed in 50% ^2H_2O, (4) glucose was converted to MAG in 50% [U-$^2H_6$]acetone, or (5) glucose was converted to MAG using $^2H_2SO_4$.

NMR spectroscopy

NMR spectroscopy was performed as previously reported [6]. Briefly, the samples were dissolved in 150 μl of acetonitrile and 10 μl of deuterium-depleted water. More water was added when necessary to completely dissolve the sample. ^1H NMR spectroscopy was performed on a 14.1 T Varian spectrometer using a 3-mm Nalorac inverse detection probe for ^1H detection. Acquisition parameters included 90° pulse excitation pulse, 1 s delay between pulses, 2 s acquisition time, and 256 acquisitions. Deuterium NMR spectra were collected using a Nalorac single-tuned deuterium probe using the following parameters: 90° excitation pulse, zero delay between pulses, 1 s acquisition time and an average of 5000–10,000 acquisitions over 2–4 h. Waltz-16 decoupling was used to remove proton–deuterium *J* coupling. Deuterium spectra were collected in blocks of 256 acquisitions without lock followed by a single pulse on the proton channel. The proton position of the solvent peak was ascertained and Z_0 was automatically adjusted as a correction to any field drift since the collection of the last block. At the end of acquisition all blocks were summed for the final spectrum. Carbon-13 spectra were collected on the same samples (after the addition of 20 μl of deuterated acetonitrile for locking) using a 3-mm broadband probe tuned to 150 MHz using the following parameters: 45° excitation pulse, zero delay between pulses, and a 3 s acquisition time provided fully relaxed carbon spectra. A Waltz-16 sequence was used to decouple protons from ^{13}C.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) of the urinary AG and other derivatives was performed on a Dionex Summit HPLC system equipped with a Vyadec C-18 column, a multichannel UV wavelength detector set at 254 nm, and a automatic sampler set to inject 5–20 μl of sample. The sample was eluted with 7% acetonitrile in water with a trace of H_2PO_3 (0.72 ml per 1 L of water).

Analysis of ^2H and ^{13}C NMR spectra

Metabolic flux data were estimated from the NMR spectra as previously reported [6,7]. The fractional contribution of glycogen to endogenous glucose production is calculated as $1 - (H5/H2)$, where H5 and H2 represent the ^2H peak area at the 5 and 2 positions of MAG, respectively. Carbon-13-isotopomer data from the C2 of MAG yield relative metabolic fluxes, with citrate synthase flux set to a value of 1.0. Relative gluconeogenic output was calculated as (C2Q − C2D23)/C2D23. Pyruvate cycling was calculated as (C2D12 − C2Q)/C2D23. Anaplerosis was calculated as (C2D12 − C2D23)/C2D23, where C2Q, C2D12, and C2D23 are the fractional contribution each multiplet makes to the total C2 resonance.

Results

Urinary AG

AG was measured in each urine sample by HPLC after treatment with urease. The average content of AG was 150 ± 75 mg and subsequent purification by SPE gave a 70–88% yield. SPE purification was chosen because it has been shown to be an effective method for purifying acetaminophen metabolites from urine samples [14]. Fig. 1 shows the ^{1}H spectrum of the urine before and after purification of AG using a SPE column (HPLC tracings shown as insets). Prior to purification, the ^{1}H spectra looked very similar to those reported in the literature [15]. Those spectra were also used to assign many of the resonances in Fig. 1. After purification, the majority of the salt and extraneous metabolites had been removed leaving acetaminophen derivatives present as ~10% acetaminophen-sulfate and 90% acetaminophen-glucuronide.

Conversion of AG to MAG

Fig. 2 shows a scheme of all of the synthetic steps used to convert AG to MAG. The conversion of AG to glucose was very good, with the greatest losses occurring in the reduction step (89–98% yield). The total yield of glucose from urinary glucuronide was 50–80%. Con-version of the resulting glucose to MAG was monitored by NMR and also gave a high yield (80%). Impurities remaining in this reaction mixture were mainly unhydrolyzed diacetone glucose and some unidentified glucose derivatives (likely, less favored isopropylidene glucose derivatives). The overall yield from urinary glucuronide to MAG was generally 40–80%, but varied from sample to sample likely due to the inherent variability in the urine matrix.

NMR spectra and metabolic profiles

Fig. 3 shows the typical ^{2}H NMR spectrum of MAG derived from urinary AG compared to MAG-derived plasma glucose from the same subject. The greater amount of material derived from the urinary glucuronide typically allowed NMR experiments to be completed 3 to 10 times faster than with plasma samples or in the same amount of time but with better signal to noise ratios. An obvious difference between the two spectra is the absence of the H6 deuterium signals in the urine spectrum. This loss is a result of the oxidation of C-6 during the in vivo synthesis of AG. When unlabeled AG was derivatized using solvents and reactants highly enriched with deuterium, ^{2}H NMR analysis of the MAG product indicated that it was not enriched above the natural abundance level
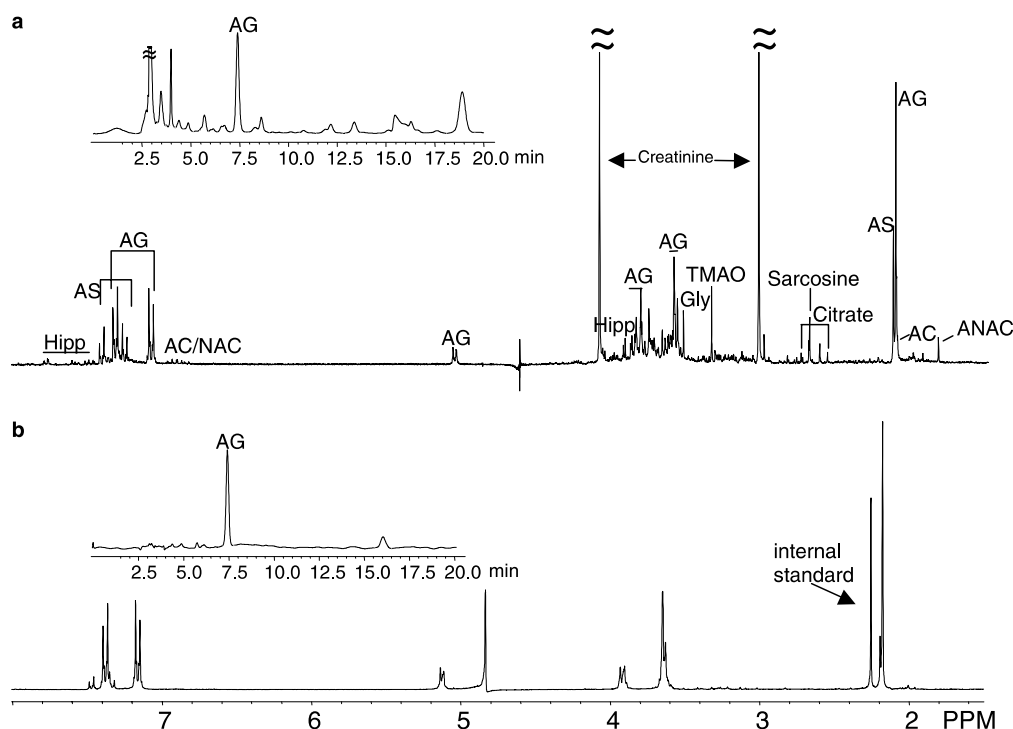

Fig. 1. (a) ^{1}H NMR spectrum of the semipurified urine of a subject after ingesting 1000 mg of acetaminophen. AC, acetaminophen cysteinyl; AG, acetaminophen glucuronide; AS, acetaminophen sulfate. The inset is a HPLC tracing. (b) The same sample after purification by C-18 chromatography. Only AG and a small amount of AS remain. (hippurate, Hipp; acetaminophen sulfate, AS; acetaminophen cysteinyl, AC; acetaminophen N-acetyl-L-cysteinyl, NAC; glycine, Gly; trimethylamin-N-oxide, TMAO).
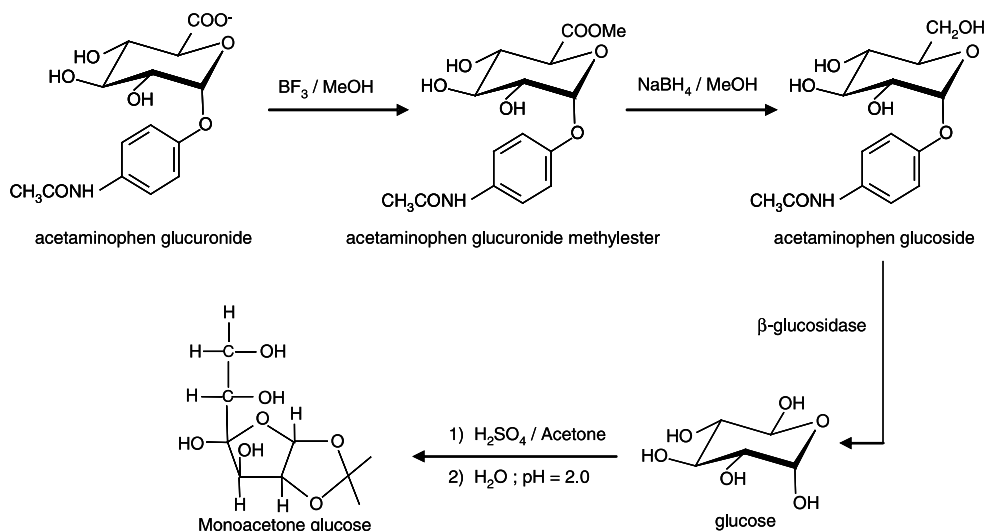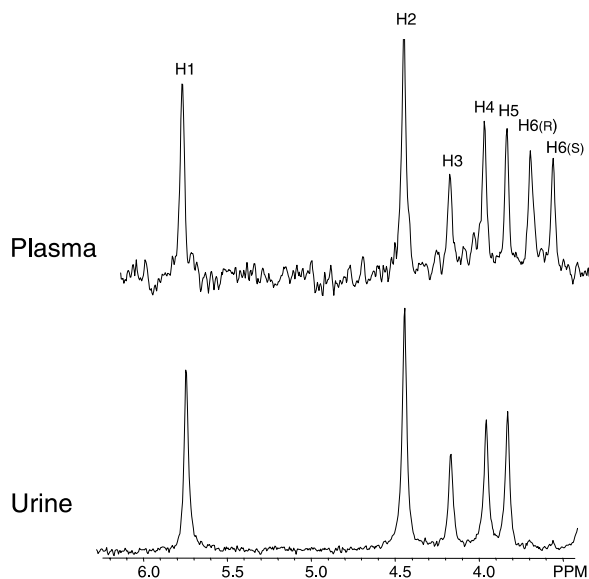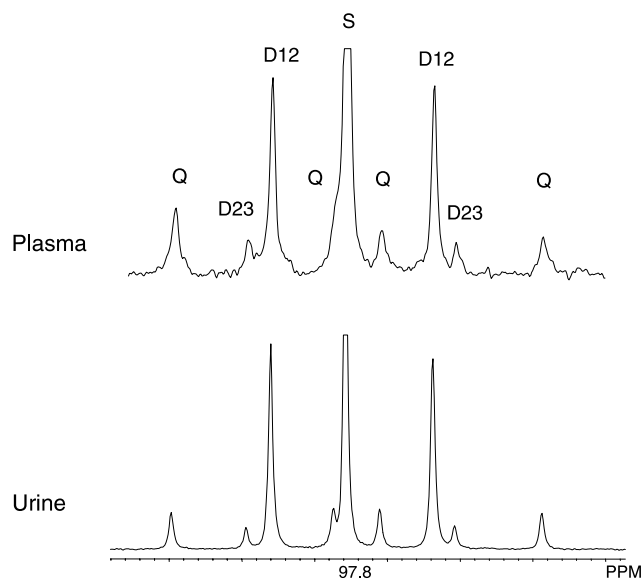
Fig. 2. Reaction scheme.

Fig. 3. ^{2}H NMR spectra of monoacetone glucose derived from both urinary glucuronide (bottom) and plasma glucose (top). Note the absence of H6 signals in the urinary glucuronide spectrum.

Fig. 4. ^{13}C NMR spectra of the C2 region of MAG derived from both urinary glucuronide (bottom) and plasma glucose (top).

in hydrogens 1–5. However, the hydrogen 6 positions were predictably enriched with deuterium following reduction in [U-^{2}H$_4$]methanol or with sodium borodeuteride.

For 5 normal volunteers after an overnight fast, the fraction of glucose derived from glycogenolysis, as determined from the ratio of H5 to H2 signal intensities from the ^{2}H spectra, was 51% ± 11. This matches the value obtained from analysis of plasma glucose of healthy subjects fasted for the same duration in a previous study [52% ± 7, Ref. 6].

Fig. 4 shows ^{13}C spectra of MAG derived from urinary AG and from plasma glucose of the same patient.

Again, the superior quality of the urine-derived MAG spectra was easy to appreciate. The two sources consistently gave identical isotopomer data and corresponding flux data as a previous report where flux data were derived from spectra of glucuronide or glucose [4]. Here, the ^{13}C NMR spectra of MAG derived from urinary AG report that gluconeogenesis is 2.44 ± 0.59, pyruvate cycling is 5.22 ± 0.45, and anaplerosis is 7.66 ± 0.80, all relative to a TCA cycle flux of 1. These values compare very well to control patients from a previous study based upon analysis of glucuronic acid (gluconeogenic output = 1.85 ± 0.31, pyruvate cycling = 5.27 ± 1.05, anaplerosis = 7.11 ± 1.13) [6].

Discussion

Since the liver is fundamental to glucose homeostasis, a simple assay for interrogating multiple pathways involved in hepatic glucose production could be an important clinical research tool. We describe a simple technique that allows a minimally invasive study of disorders of glucose homeostasis, such as fasting hyperglycemia in type II diabetes. We have previously shown that a liver flux profile (LFP) can be obtained by using a combination of stable isotope tracers and NMR [6]. The method is simple and is only limited by the amount of tracer that can be safely administered and sample availability. The technique described herein represents a significant advancement for general clinical implementation of a NMR-derived LFP, as urine collection is noninvasive and does not require large volume phlebotomy.

Urinary AG has been used as a surrogate of hepatic glucose, but only in conjunction with carbon tracers [8,9,11,12]. To our knowledge, there are no other reports of the use of glucuronides for monitoring enrichment of hepatic glucose from ^2H_2O. One limiting feature of the ^2H NMR spectrum of AG is the poor chemical-shift dispersion. As demonstrated here, conversion of AG to MAG alleviates this problem and allows a direct measure of the ^2H enrichment at each site in MAG.

The yield for the conversion of urinary AG to glucose was 50–85%, generally higher than previously reported values (40%) [8]. The broad range in yield seemed to correlate with the amount of AG in urine, with the highest yields coming from urine samples that contained less than 100 mg of AG. The use of larger SPE columns with higher capacities would likely increase yields, but this was not attempted.

Exchange studies were carried out to investigate whether or not deuterium enrichment is modified during the multistep synthesis of MAG from AG. While not an issue for ^{13}C studies, protons could possibly exchange during conversion and lead to erroneous results for the ^2H analysis. However, even in reactions using 50% deuterated reagents and solvents (methanol, water, sodium borohydride, acetone) no significant exchange was observed. An exception was at the H6 position where ^2H labeling from d_4-methanol and sodium borodeuteride was observed. This result was expected since glucuronide C6 is reduced by sodium borohydride in methanol during the conversion of the glucuronide methyl ester to the glucoside analog.

While C6 deuterium enrichment in plasma glucose is indicative of glucose production originating in the TCA cycle [2], no such information can be deduced from MAG derived from AG. Without this information, one cannot distinguish gluconeogenesis from glycerol versus that from the TCA cycle. Nevertheless, the contribution of glycogen to glucose production can be obtained from urinary AG and this has been shown to differ in humans

during a number of different pathological states including diabetes [16–19], obesity [19], and cirrhosis [20]. Isolation and conversion of AG to a readily analyzable NMR sample in high yield and purity now offer the possibility of taking these measurements in a much broader range of patient populations.

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