

## Proton nuclear magnetic resonance spectroscopic determination of ethanol-induced formation of ethyl glucuronide in liver

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### Abstract

Ethyl glucuronide (ethyl- $\beta$ -D-6-glucosiduronic acid, EtG), a unique metabolite of ethanol, has received much recent attention as a sensitive and specific biological marker of ethanol consumption. Formed in the liver via conjugation of ethanol with activated glucuronate, EtG remains detectable in serum, plasma, and hair for days after ethanol abuse. Thus far, gas chromatography–mass spectrometry and enzyme-linked immunosorbent assays have been developed to detect trace quantities of EtG for forensic purposes, but reports of the nuclear magnetic resonance (NMR) properties of EtG have been scarce. Herein we present the first report of EtG determination using proton NMR spectroscopy. We collected 700-MHz proton spectra of liver extracts from rats treated with a 4-day binge ethanol protocol (average ethanol dose: 8.6 g/kg/day). An unexpected signal (triplet, 1.24 ppm) appeared in ethanol-treated liver extracts but not in control samples; based on chemical shift and multiplicity, we suspected EtG. We observed quantitative hydrolysis of the unknown species to ethanol while incubating our samples with  $\beta$ -glucuronidase, confirming that the methyl protons of EtG were responsible for the triplet at 1.24 ppm. This study demonstrates that proton NMR spectroscopy is capable of detecting EtG and that future NMR-based metabolomic studies may encounter this metabolite of ethanol.

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Ethyl glucuronide (ethyl- $\beta$ -D-6-glucosiduronic acid, EtG)<sup>2</sup> is a minor direct metabolite of ethanol that has received much recent attention as a sensitive and specific biological marker of ethanol consumption. Due to interest in developing methods capable of detecting minute quantities of this compound for forensic applications [1–3], efforts have focused on gas chromatography–mass spectrometry [4–6] and even enzyme-linked immunosorbent assay techniques [7], but the nuclear magnetic resonance (NMR) properties of EtG have not been reported to our knowledge. In the emerging field of metabolomics, NMR spectroscopy is a prominent tool for the analysis of

compounds found in biofluids and tissue extracts [8–10] because it is capable of detecting many classes of molecules in the same sample without preselection of analytes [11]. Moreover, the sensitivity of NMR has increased dramatically in recent years due to technical advances such as cold probes that increase the signal to noise ratio, small-volume sample tubes that allow the detection of smaller amounts of material, and improved digital electronics that increase the effective dynamic range of the technique. These advances extend the detection limit for NMR and thus increase the number of compounds that may be identified during experiments and screening tests. As the number of detected compounds grows, it will be of great use to have access to publications that give NMR spectral properties for molecules that have not yet received great attention. Therefore, we present what is to our knowledge the first published report describing the detection of ethyl glucuronide by pro-

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<sup>2</sup> Abbreviation used: EtG, ethyl glucuronide.

ton NMR spectroscopy. We hope that the presentation of this information will facilitate the correct interpretation of NMR data from future clinical and animal metabolomic studies in which ethanol intake is a possibility.

## Materials and methods

### Animals

Adult male Sprague–Dawley rats (275–325 g) were treated with a modified 4-day binge ethanol protocol as previously described [12]. A 15% (w/v) ethanol Reitz High Fat diet was used with an appropriate isocaloric control. Male Sprague–Dawley rats (250–300 g) were given an initial dose (5 g/kg, 25% w/v, in a vehicle of nutritionally complete diet) with subsequent doses determined using a six-point behavior scale [13]. Control animals received a diet equal to the average of that of all ethanol-treated animals. All animals had free access to water throughout the experiment. This protocol produces physical dependence to ethanol [13] and induces neuropathology modeling that found in human alcoholics [12]. Blood ethanol concentration was measured using electrochemical detection of an enzymatic reaction with an Analox Instruments Model GM-7 analyzer (Analox Instruments, MA). Ethanol groups had mean blood levels around 300 mg/dl (range 250–375 mg/dl). Within 1 h after the last treatment, all animals were sacrificed, and liver samples were collected and stored at  $-80^{\circ}\text{C}$  until further analysis. All protocols in this study were approved by the Institutional Animal Care and Use Committee and were in accordance with National Institute of Health regulation for the care and use of animals in research.

### Tissue extraction and sample preparation

Liver tissues were removed from the  $-80^{\circ}\text{C}$  freezer and placed in liquid nitrogen. Tissues were then pulverized with a stainless steel mortar and pestle and extracted with perchloric acid [14]. The extracts were lyophilized, dissolved in  $\text{D}_2\text{O}$  containing 3-(trimethylsilyl)propionic-2,2',3,3'- $\text{d}_4$  acid (TSP,  $\delta = 0$  ppm) and formate (as frequency and concentration references, respectively), and adjusted to pH 7.0 (pD 6.6) with DCl or NaOD prior to NMR analysis.

### Enzymatic hydrolysis

To confirm the presence of a glucuronide, we performed an enzymatic hydrolysis by adding 2500 IU  $\beta$ -glucuronidase (E.C. 3.2.1.31) [15] from *Helix pomatia* crude extract (MP Biomedicals, Inc., Irvine, CA). The  $\alpha$ -anomers of glucuronide conjugates are energetically unfavorable, and therefore the  $\alpha$ -anomers typically are not present. We acquired “preincubation” proton spectra after adjusting the pH of the liver extracts to 5.0 (pD 4.6) for optimal activity of the *H. pomatia* enzyme using 7 M DCl. After acquiring a pre-incubation spectrum, enzyme was added

to the liver extract samples, and the samples were incubated overnight in a  $37^{\circ}\text{C}$  water bath. As a positive control, methyl glucuronide (Sigma, St. Louis, MO) was incubated with  $\beta$ -glucuronidase under the same conditions.

### NMR spectroscopy

Initial studies were conducted on a Varian Inova 700-MHz spectrometer. Proton spectra were acquired in fully relaxed mode (duty cycle = 45 s), at  $25^{\circ}\text{C}$ , with 1 s presaturation of water protons immediately prior to a  $90^{\circ}$  excitation pulse. Other parameters were as follows: 16 transients, spectral width 8600 Hz, 64 k data points. Hydrolysis of EtG by  $\beta$ -glucuronidase was monitored on a Varian Inova 400-MHz spectrometer. Spectra collected before and after overnight incubation were acquired at  $25^{\circ}\text{C}$  with 45 s duty cycle, 1.5 s presaturation of water protons,  $90^{\circ}$  excitation pulse, 8 transients, 4000 Hz spectra width, and 16 k data points. Sequential spectra acquired during incubation with  $\beta$ -glucuronidase were acquired at  $37^{\circ}\text{C}$ , with identical parameters except that 32 transients were acquired for each spectrum. Routine spectral analysis was accomplished using software written in-house in the Matlab (The MathWorks, Inc., Natick, MA) programming environment (PC Nicholas). Peak fitting was accomplished using the ACD/SpecManager 1D NMR Processor (Advanced Chemistry Development, Inc. Toronto, Ontario, Canada).

### Ethyl glucuronide standard

A sample of EtG (Aurora Analytics, Baltimore, MD) was obtained to validate spectral assignments.

## Results and discussion

Analysis of 700-MHz proton spectra of liver extracts revealed an unexpected triplet at  $1.2380 \pm 0.006$  ppm ( $J = 7.11 \pm 0.05$  Hz) (data reported as means  $\pm$  standard deviation of 11 samples). This triplet was present in all spectra of liver extracts from animals that received ethanol ( $n = 11$ , see Figs. 1a and g–k for representative spectra) and absent from control samples ( $n = 11$ , see Figs. 1b–f). The multiplicity of this signal suggested the presence of an ethyl group, but the chemical shift was inconsistent with ethanol, which was confirmed by adding a small amount of ethanol to the sample, reacquiring data at 400 MHz, and observing a distinct triplet at 1.19 ppm (Fig. 2c).

Based on the presence of an ethyl group associated with exposure to large doses of ethanol, we suspected that the unassigned triplet was due to the methyl protons of ethyl glucuronide ( $\text{C}_8\text{H}_{14}\text{O}_7$ , see Fig. 2d for structure). We searched our 700-MHz spectra for signals due to the  $\beta$ -anomeric proton of EtG and discovered a doublet at  $4.4888 \pm 0.0094$  ppm ( $J = 7.95 \pm 0.16$  Hz), the intensity of which appeared to be correlated to that of the putative EtG triplet at 1.24 ppm (Figs. 1g–k). Based on the results of peak fitting of these signals in five 700-MHz spectra,

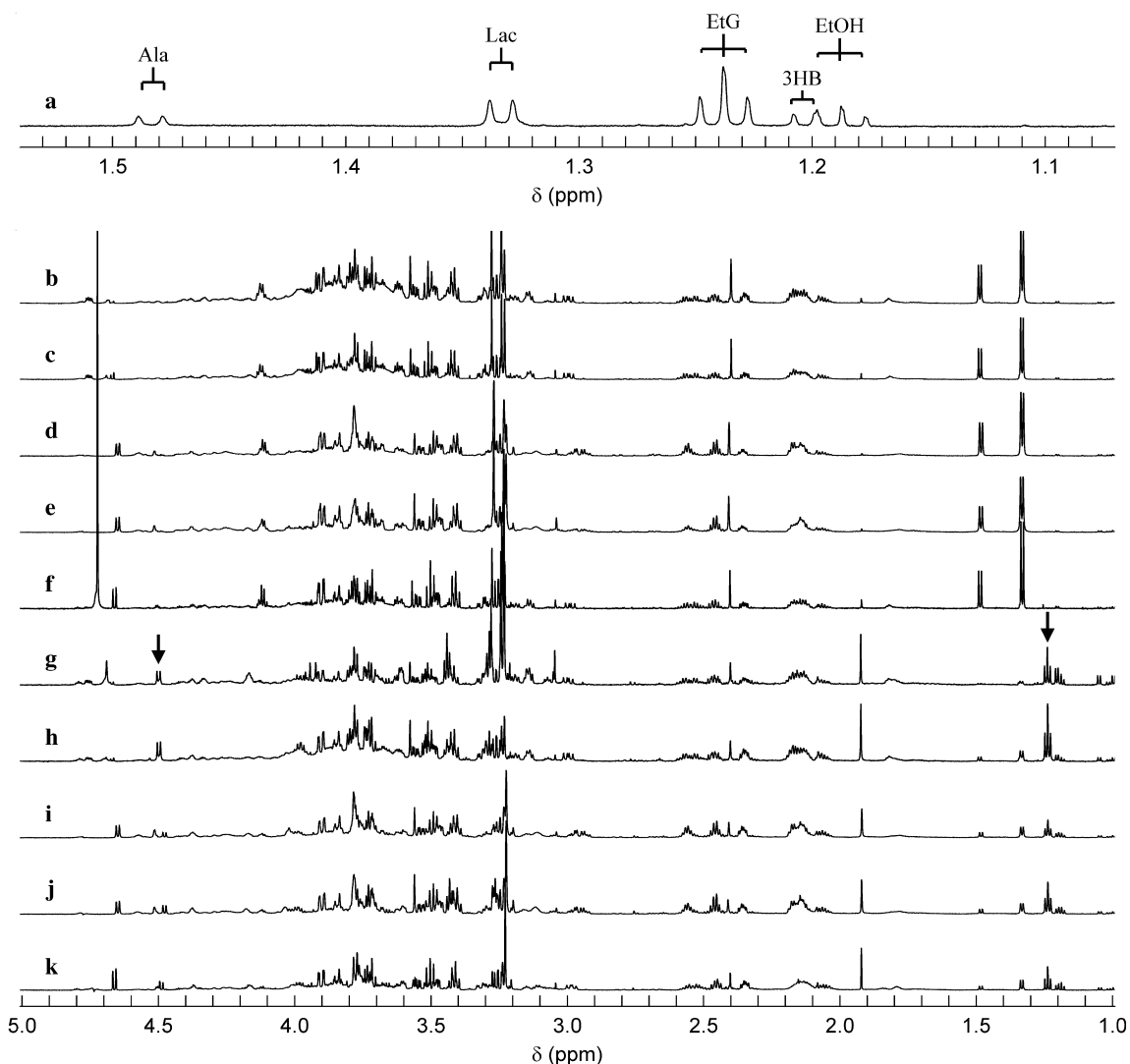


Fig. 1. Expanded 700-MHz  $^1\text{H}$  spectrum (a) of liver extract from an animal treated with ethanol shows a triplet at 1.24 ppm which was later confirmed to be due to ethyl glucuronide. This signal is not due to ethanol, which resonates at 1.19 ppm (see Fig. 2c). The triplet was absent from spectra of liver extracts from animals that were not given ethanol (b–f). In ethanol-treated animals (g–k), the intensity of the triplet at 1.24 ppm appears to be correlated with the intensity of the doublet at 4.49 ppm with a ratio of approximately 3:1, consistent with the triplet and the doublet originating from the methyl and anomeric protons of EtG, respectively. Abbreviations: alanine, Ala; ethanol, EtOH; ethyl glucuronide, EtG;  $\beta$ -hydroxybutyrate, 3HB; lactate, Lac.

the ratio of the triplet to doublet signal was  $3.03 \pm 0.66$ , consistent with the assignment of the doublet to the anomeric proton of EtG. We attribute the variance to (i) quantification of very small signals on an imperfect baseline and (ii) inconsistencies in the effect of the presaturation sequence on signals near the water resonance across different spectra.

Comparison of spectra from a liver sample acquired before (Fig. 2a) and after (Fig. 2b) overnight incubation with  $\beta$ -glucuronidase reveals hydrolysis of EtG to ethanol + glucuronate in a liver extract from an ethanol-treated animal. Incubation with  $\beta$ -glucuronidase resulted in decreased intensity of the triplet at 1.24 ppm (EtG methyl group) and the doublet at 4.49 ppm (EtG anomeric proton), with a corresponding increase in the intensity of the triplet at 1.19 ppm (ethanol). The identity of the triplet at 1.19 ppm was confirmed by observing an increased

intensity in this triplet in a spectrum acquired after adding ethanol directly to the samples (Fig. 2c).

We performed several controls to validate our procedures. To rule out the possibility that incubation at 37 °C and pH 5 caused nonspecific formation of ethanol, we incubated one extract from ethanol-treated liver without addition of  $\beta$ -glucuronidase, and as expected no ethanol formation or EtG hydrolysis were observed (Figs. 3a and b). This finding shows that the reaction observed in Figs. 2a and b was catalyzed by  $\beta$ -glucuronidase. In addition we incubated one control (non-ethanol-treated) liver extract which contained no detectable EtG (Fig. 3c) in the presence of  $\beta$ -glucuronidase to demonstrate that addition of enzyme and overnight incubation do not cause ethanol to appear in the spectra (Figs. 3c and d). This finding demonstrates that (i) the reaction in Figs. 2a and b required the source of the unassigned triplet at 1.24 ppm as a

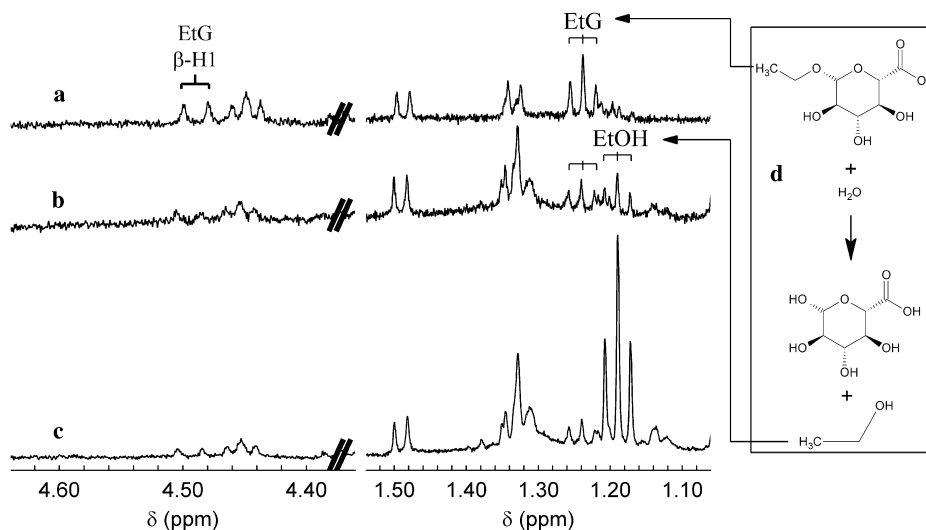


Fig. 2. Identification of ethyl glucuronide in proton NMR spectra of liver extracts. The 400-MHz  $^1\text{H}$  spectra acquired before (a) and after (b) overnight incubation with  $\beta$ -glucuronidase show decreased intensity of the EtG methyl triplet (1.24 ppm) and the anomeric doublet (4.49 ppm), and formation of ethanol. Spectrum (c) from the same sample after addition of pure ethanol, which resonates at 1.19 ppm, confirms that the triplet at 1.24 ppm is not ethanol and that ethanol is the product of the reaction. (d) Shows the hydrolysis of EtG to glucuronate + ethanol catalyzed by  $\beta$ -glucuronidase.

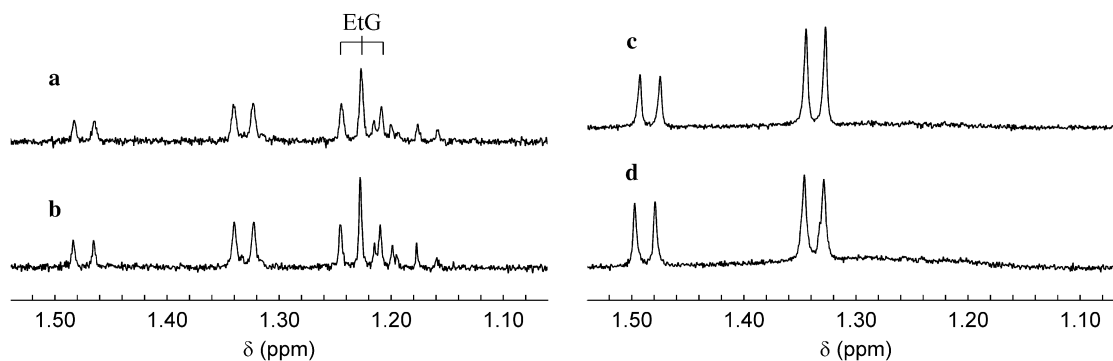


Fig. 3. Spectra acquired before (a) and after (b) overnight incubation of an EtG-containing liver extract without addition of  $\beta$ -glucuronidase show no evidence of either decreased EtG intensity or formation of ethanol, demonstrating that the reaction in Figs. 2a and b is due to enzymatic activity rather than nonspecific degradation. Spectra of an extract from control liver that contained no EtG acquired before (c) and after (d) overnight incubation with  $\beta$ -glucuronidase show no formation of ethanol due to incubation, demonstrating a specific requirement for the species that gives rise to the triplet at 1.24 ppm as a substrate for the production of ethanol.

substrate and (ii) there was no contamination of the  $\beta$ -glucuronidase with ethanol. Next, we recorded sequential 400-MHz proton spectra at 37 °C (Fig. 4a) to demonstrate the stoichiometry during the enzymatic hydrolysis of EtG to ethanol. By integrating the baseline-corrected signals from ethanol and EtG and normalizing by the integral for formate (which had been added as an exogenous concentration reference [16]), we were able to demonstrate that the sum of [ethanol] + [EtG] was approximately constant over the 49 h during which the data were recorded (Fig. 4b). This finding is consistent with the hydrolysis of EtG to form ethanol, and provides important support for our assignment of the triplet at 1.24 ppm to EtG. In addition, during the hydrolysis of EtG, we were able to observe an increase of glucuronic acid by monitoring the  $\alpha$ -H1 resonance at 5.22 ppm. Glucuronic acid exists as  $\alpha$ - and  $\beta$ -anomers in solution, but at 37 °C the residual water sig-

nal overlaps with the  $\beta$ -H1 signal at 4.62 ppm, and thus the signal from the  $\beta$ -anomer cannot be quantified reliably. During the 49 h incubation, the increase in signal from the  $\alpha$ -H1 proton was several times greater than the increase in the ethanol signal, when adjusted for the number of protons contributing to each resonance. Whereas we observed a quantitative increase in signals from  $\alpha$ - and  $\beta$ -anomers of free glucuronate during hydrolysis of methyl glucuronide (a positive control, data not shown), it is likely that the large increase in signal from free glucuronate in liver extracts reflects hydrolysis of glucuronides other than EtG that were present in liver. Nevertheless, increased signal due to free glucuronic acid is expected during hydrolysis of EtG and provides additional confirmation that our unknown signal is caused by EtG.

Finally, a 400-MHz proton spectrum of pure EtG (Fig. 5) helped to secure our assignments; the list of

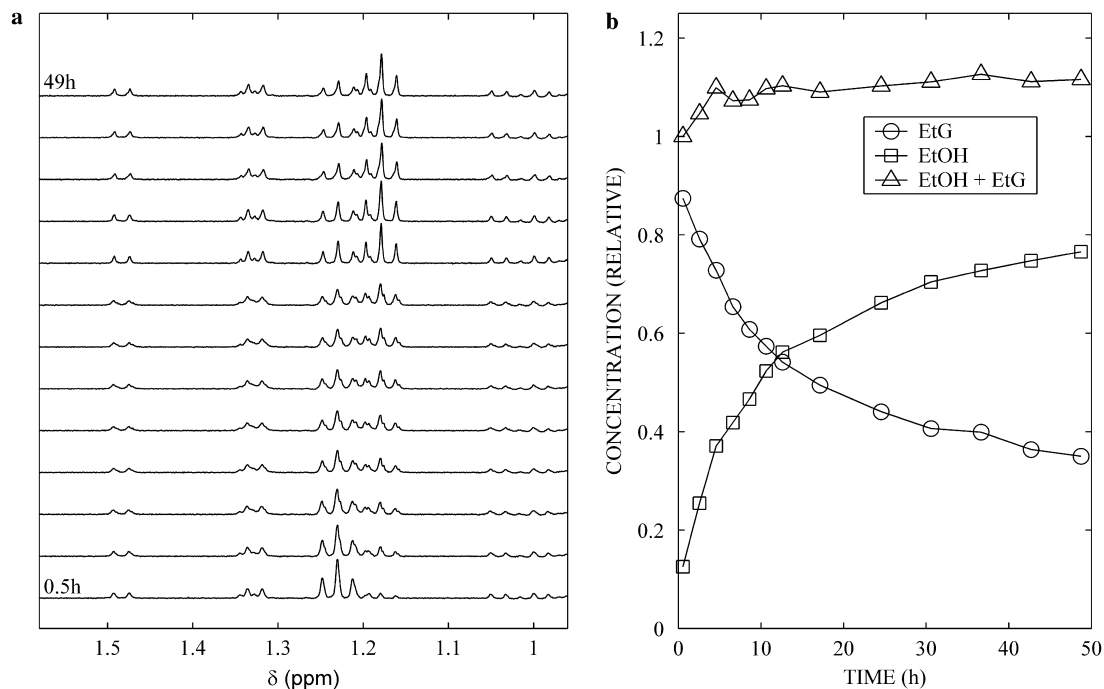


Fig. 4. (a) Sequential 400-MHz proton NMR spectra of a single sample incubated with  $\beta$ -glucuronidase at 37 °C. Over time the intensity of EtG (triplet, 1.24 ppm) decreases while the intensity of ethanol (triplet, 1.19 ppm) increases, consistent with hydrolysis of EtG. (b) Relative concentrations of ethanol and EtG over time. The sum of ethanol + EtG (triangles) is approximately constant over time, as would be expected during hydrolysis of EtG to ethanol.

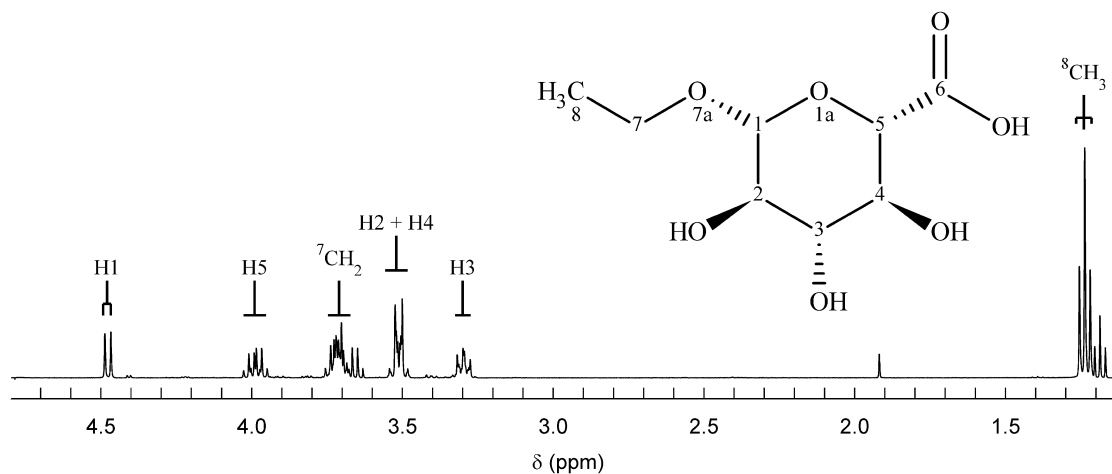


Fig. 5. The 400-MHz  $^1\text{H}$  spectrum of EtG in  $\text{D}_2\text{O}$  at pH 7, referenced to TSP at 0 ppm. Signals are assigned to the protons numbered as shown on the structure (inset). Signals from trace amounts of ethanol (1.19 ppm, t,  $J = 7.1$  Hz, 3H; 3.66 ppm, q,  $J = 7.1$  Hz, 2H) and acetate (1.92 ppm, s) were noted, but these do not interfere with the analysis of the EtG spectrum (see Table 1 for assignments). Of particular significance in the spectrum of pure EtG are the triplet at 1.24 ppm and the doublet at 4.48 ppm, both of which are entirely consistent with the presence of EtG in our spectra from extracts of liver tissue.

resonances is presented in Table 1, and these are completely consistent with our interpretation of the enzymatic hydrolysis results.

Previous NMR-based metabolomic studies have identified a triplet at 1.24 ppm. Teague et al.[17] report the detection of ethyl glucoside in the urine of human volunteers who had recently consumed rice wine and sake. Ethyl glucoside, unlike EtG, is not a metabolite in humans [17]. Rather, it is the fourth most abundant constituent of sake [18], formed during the production of this beverage [19].

Ethyl glucoside is structurally similar to EtG, with the exception that the carbonyl is reduced to a methylene group, and the two new protons give rise to a doublet of doublets at 3.86 ppm that is not present in EtG. The methyl group on ethyl glucoside would give a triplet at nearly the same chemical shift as the one on EtG (1.24 ppm). However, (i) we do not find a doublet of doublets at 3.86 ppm in our 700-MHz spectra of liver extracts (Fig. 1), (ii) we found evidence of hydrolysis by  $\beta$ -glucuronidase, (iii) we found the signal from an anomeric proton at

Table 1  
Ethyl glucuronide resonances observed in 400-MHz  $^1\text{H}$  NMR spectrum in  $\text{D}_2\text{O}$  at pH 7

| $^1\text{H}$ $\delta$ (ppm) | Multiplicity ( $J$ )    | Assignment                  |
|-----------------------------|-------------------------|-----------------------------|
| 1.24                        | t ( $J^{7,8} = 7.1$ Hz) | $^8\text{CH}_3$             |
| 3.30                        | m                       | $^3\text{CH}$               |
| 3.52                        | m                       | $^2\text{CH} + ^4\text{CH}$ |
| 3.71                        | m                       | $^7\text{CH}_2$             |
| 3.99                        | m                       | $^5\text{CH}$               |
| 4.48                        | d ( $J^{1,2} = 8.0$ Hz) | $^1\text{CH}$               |

4.49 ppm, whereas the anomeric proton of ethyl glycoside resonates at 4.93 ppm [17], and (iv) ethyl glucoside is not known to be a metabolite of ethanol. Thus, we conclude that EtG rather than ethyl glucoside is the source of the triplet observed in our liver samples.

Correct attribution of NMR spectral peaks to the molecules in a sample that give rise to these signals is crucial to the proper interpretation of experimental results. Nevertheless, the process of signal assignment is usually the most time-consuming and error-prone aspect of data analysis. For example, a previous 400-MHz  $^1\text{H}$  NMR study of liver extracts from ethanol-treated rats (36% ethanol liquid diet for 1.5 months) attributed a triplet at 1.21 ppm (referenced relative to the acetate singlet at 1.92 ppm at pH 7.5) to residual ethanol that had not evaporated during lyophilization [20], yet visual examination of the published spectra reveals the presence of weak signals (possibly a triplet) upfield of the triplet that the authors concluded was due to ethanol. While it is possible that the assignment was correct, this pattern of a weak triplet (ethanol) immediately upfield from a stronger one (EtG) (evident from our 700-MHz data, Fig. 1a) suggests that the triplet previously observed is consistent with EtG. Therefore, taken together, the data that we present suggest that EtG is detectable by proton NMR spectroscopy in liver of ethanol-exposed animals, that EtG may in fact have been detected (though unrecognized) in previous studies, and that signals from this biomarker for ethanol administration will likely be encountered in future NMR studies of liver. Other studies have found EtG in plasma, urine, hair, and other tissues [21], suggesting that EtG produced in liver is distributed throughout much of the body, where it may persist for some time. Serum and urine EtG concentrations in the 10–600  $\mu\text{M}$  (2.2–132 mg/L) range have been measured under various conditions [21]. While proton NMR spectroscopy would not be an optimal method for detecting EtG at the lower end of that range, if a sample contained 0.5 mM EtG or more, we would expect to find at least the triplet at 1.24 ppm in the proton NMR spectrum. On the other hand, metabolomic studies often benefit from analysis of tissue samples in addition to biofluids in animals [22–24] including humans [25], and our data suggest that NMR spectroscopic analysis of liver extracts can detect the presence of EtG following high-dose ethanol.

The present study highlights the benefits of NMR spectroscopy with minimal preselection of analytes as an

unbiased method for the discovery of novel metabolites. Though other analytical methods certainly possess superior sensitivity, they require prior knowledge of analytes, and therefore EtG (an unexpected compound) would likely have gone undetected. NMR spectroscopy not only alerted us to the presence of an unexpected compound, but also provided structural information that aided us in its identification.

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