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1D Proton NMR Spectroscopic Determination of Ethanol and Ethyl Glucuronide in Human Urine

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Forensic and legal medicine require reliable data to indicate excessive alcohol consumption. Ethanol is oxidatively metabolized to acetate by alcohol dehydrogenase and non-oxidatively metabolized to ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanol, or fatty acid ethyl esters (FAEE). Oxidative metabolism is too rapid to provide biomarkers for the detection of ethanol ingestion. However, the non-oxidative metabolite EtG is a useful biomarker because it is stable, non-volatile, water soluble, highly sensitive, and is detected in body fluid, hair, and tissues. EtG analysis methods such as mass spectroscopy, chromatography, or enzyme-linked immunosorbent assay techniques are currently in use. We suggest that nuclear magnetic resonance (NMR) spectroscopy could be used to monitor ethanol intake. As with current conventional methods, NMR spectroscopy doesn't require complicated pretreatments or sample separation. This method has the advantages of short acquisition time, simple sample preparation, reproducibility, and accuracy. In addition, all proton-containing compounds can be detected. In this study, we performed ^{1}H NMR analyses of urine to monitor the ethanol and EtG. Urinary samples were collected over time from 5 male volunteers. We confirmed that ethanol and EtG signals could be detected with NMR spectroscopy. Ethanol signals increased immediately upon alcohol intake, but decreased sharply over time. In contrast, EtG signal increased and reached a maximum about 9 h later, after which the EtG signal decreased gradually and remained detectable after 20-25 h. Based on these results, we suggest that ^{1}H NMR spectroscopy may be used to identify ethanol non-oxidative metabolites without the need for sample pretreatment.

Key Words : Nuclear magnetic resonance (NMR), Metabolomics, Ethanol, Ethyl glucuronide (EtG), Multivariate analysis

Introduction

Ethanol is a widely consumed drink and drug. Ethanol overdose, however, causes alcohol-related diseases (*e.g.* liver disease, coronary artery disease, *etc*) and many social problems such as drunk driving, reduced productivity, and violent behavior. Analytical tools are needed for monitoring alcohol consumption to reduce these health and social problems.

The majority of ingested alcohol (95-98%) is digested, while the remaining 2-5% is excreted.[1] Alcohol metabolism passes through 2 pathways. Most consumed alcohol is digested via oxidative metabolism in the liver, where ethanol is converted to aldehyde and acetic acid. Through non-oxidative metabolism, ethanol is converted to ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanol, or fatty acid ethyl esters (FAEE).[2]

The traditional method used to prove recent alcohol intake is to measure the ethanol concentration in the body. The direct detection of alcohol, however, is not proper method for testing that occurs long after consumption, because alcohol is quickly metabolized over about 5 h.[3] Furthermore, small amounts of alcohol can be present absent consumption. An alcohol biomarker other than ethanol is needed. The biomarker should provide information about the drinking pattern. Several traditional alcohol biomarkers are already known, such as gamma glutamyl transferase (GGT), aspartate amino transferase (AST), carbohydrate-deficient transferrin (CDT), ethyl sulfate (EtS), phosphatidyl ethanol (PEth), *etc.*[4] These biomarkers are affected by sex, age, hormonal status, *etc*. For this reason, a new, more powerful alcohol biomarker must be developed. EtG, the minor metabolite of ethanol, is a good biomarker candidate because it is stable,[5] non-volatile, water soluble, highly sensitive,[6] and is detectable in body fluid, hair,[7-10] and tissues.[11] EtG is used to monitor acute and chronic alcohol intake whereas CDT, GGT, and others are used for the detection of chronic alcohol abuse. Furthermore, EtG is formed only after ethanol is consumed and can be detected in urine 70 or more hours after ethanol elimination is complete.[12] Because of these advantages, EtG analysis in urine is widely used without the need for transport or storage of samples.

EtG analysis is carried out mainly by mass spectroscopy coupled with chromatography. Nishikawa *et al.* carried out EtG analysis in human serum by liquid chromatography-electrospray ionization mass spectrometry.[13] However, this method is typically used to analyze a single target material or chemical and requires sample processing prior to analysis.

Current mass spectroscopy methods are also laborious and expensive. To overcome these inconveniences, the field of NMR-based metabolomics has been introduced. The field of metabolomics seeks to understand biological pathways from metabolite changes and is applied to toxicology, medical science, phytochemistry, forensic science, *etc*. Metabolomics employs analysis tools such as NMR spectroscopy, mass spectroscopy, and high-performance liquid chromatography.

NMR, the most important method in metabolomics, has the advantages of short acquisition time, simple sample preparation, non-destructive sampling, and accuracy. Additionally all proton-containing compounds can be detected and analyzed. In 2004, Teague *et al.* confirmed ethyl glucoside (the other minor metabolite of ethanol, not EtG) in human urine by NMR.[14] Human urine samples were treated with sodium buffer solution (pH 7.4) and centrifuged for 10 min. Also, Nicholas *et al.* carried out NMR analyses of EtG in rat liver samples and blood serum in 2006[15] and 2008,[11] respectively. In their experiments, liver tissue and blood were collected from ethanol-treated and control rats. Liver tissue was extracted by perchloric acid and blood serum was treated with a neutral buffer (pH 7.0). Samples were analyzed by Varian Inova 700 MHz NMR. EtG concentration increased with elimination of ethanol. Note that these authors described the use of preprocessing methods, such as adding buffer solution, extraction, and centrifugation.

The goal of this study was to characterize ethanol metabolism by measuring ^{1}H NMR spectra with untreated samples. Also, we suggest this method may be applied in the forensic field whether alcohol is taken or not by someone. We show that NMR-based metabolomics provide results consistent with previous data.

Materials and Methods

Sample Collection. Urine samples were collected over time from 5 healthy male volunteers, 20-25 years old, on normal diets, after they had consumed a bottle of So-ju (a Korean alcoholic beverage, 360 mL) and 1 L of drinking water. A bottle of So-ju contains 57 g of ethanol. Urine samples were collected about 46 h after initiation of consumption; Table 1 describes the details of collection times for each subject. Sodium fluoride (NaF, 1% w/v) was added to the samples to prevent perturbation. Samples were kept refrigerated at 4 °C because EtG concentration in urine is affected by *Escherichia coli*, the source of β-glucuronidase.[16]

Preparation of Urinary Samples for NMR Analysis. 450 μL of urinary sample was mixed with 50 μL of 20 mM 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid, sodium salt (TSP-d_4) dissolved in 99.9% deuterium oxide; final TSP concentration was 2 mM. These samples were transferred into 5 mm NMR tubes, which were stored at 4 °C except during NMR analysis.

NMR Spectroscopy. Proton NMR spectroscopy was carried out on a 500 MHz Varian Unity-Inova (Varian Inc., Palo Alto, CA) operating at a proton frequency of 499.789 MHz. All instruments were equipped with an ID/PFG 5 mm probe at a temperature of 298 K. Deuterium oxide and TSP-d_4 were used as the field-lock frequency and chemical shift reference, respectively. Water signal was suppressed by presaturation pulse sequence. Each ^{1}H NMR spectrum was recorded over 512 scans requiring about 30 min total acquisition time. All data was referenced to TSP-d_4 at δ 0.00 ppm and was apodized with an exponential function using a line broadening of 0.2 Hz.

NMR Data Processing and Analysis. Acquired ^{1}H NMR spectra were Fourier-transformed, phased, referred manually by VnmrJ (Varian Inc., USA). All NMR spectra were imported into Chenomx NMR Suite 6.01 software (Chenomx Inc., Canada) and spectral assignment was performed using this software and published literature data. We used the δ 0.5-9.0 ppm region of spectra for peak assignment. The residual proton signals corresponding to water and urea resonances (δ 4.6-6.7 ppm) and TSP-d_4 (δ 0.0 ppm) were removed from the native data.

For quantitative analysis, the areas of the NMR peaks corresponding to EtG and ethanol resonances were integrated using Mnova 5.3.1 (Mestrelab Research, USA). The intensity for these peaks in a given spectrum was calculated and then normalized to TSP.

The first step of general process for multivariate analysis in metabolomics study is spectral data binning. Spectral binning is a common data reduction technique used for high-throughput data pre-processing. It is used to prepare spectra for multivariate analysis. The ^{1}H NMR spectra were binned using Chenomx NMR Suite 6.01 software (Chenomx Inc., Canada). The spectral region δ 1.22-1.3 ppm and δ 4.3-4.56 ppm correspond to EtG signals was segmented into regions of 0.01 ppm width giving a total of 811 integrated regions per NMR spectrum. Then, we imported binned files into and investigated multivariate analysis by the statistical analysis software SIMCA-P+ 12.0 (Umetrics, Sweden) to identify classification of all samples. To visualize metabolic differentiation in urine, the pattern recognition methods were

Table 1. Urine sample collection times. 0 h: pre-dose sample

	Urine Sample Collection Times									
Man 1	0 h	2 h	6 h 20 m	9 h 20 m	14 h	20 h 20 m	25 h			
Man 2	0 h	1 h 40 m	3 h 40 m	6 h 30 m	8 h	9 h	13 h	24 h		
Man 3	0 h	1 h	1 h 30 m	2 h	4 h 50 m	7 h 30 m	9 h	12 h	14 h 40 m	21 h 50 m
Man 4	0 h	2 h	3 h 40 m	5 h 10 m	7 h 20 m	9 h 20 m	13 h	19 h 40 m	29 h	
Man 5	0 h	42 m	1 h 10 m	2 h 40 m	4 h 45 m	6 h 40 m	9 h	12 h	20 h 50 m	

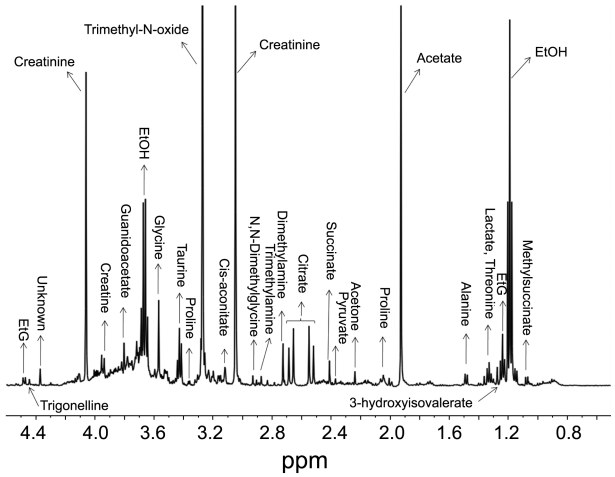

Figure 1. NMR spectrum of the 7 h 30 min sample from volunteer #4 was selected as an example for the assignment of metabolites, and assignments were made using the ChenomX 500 MHz library database and a literature compilation.

applied to NMR spectral data.

Results

Assignments of Urine Samples. Forty-three samples were available for this study. Assignment was processed using the ChenomX 500 MHz library database and a literature compilation.[17-19] Figure 1 shows the assigned spectrum at 7 h 30 min from assignment of all metabolites and TSP-d_4 (δ 0.00 ppm), water, and urea resonances (δ 4.4-6.2 ppm) were removed. We verified EtG and ethanol signals, and other common urine metabolites such as amino acids, 3-hydroxybutyrate, creatine, creatinine, creatine phosphate, trimethyl-*N*-oxide, guanidoacetate, cis-aconitate, pyruvate, succinate, betaine, taurine, lactate, acetate, *etc*.

Determination of Ethanol and EtG in ^{1}H NMR Spectrum. For each data set, ethanol and EtG signals are absent from 0 h samples (before alcohol consumption). In some cases, Ethanol could be detected because this compound is commonly present in body without alcohol intake. However, ethanol and EtG present originally in subject's bodies were not apparent in the 500 MHz NMR spectrum, so subsequent spectra provide reliable data for these signals.

Around 3 h, EtG signals appeared at δ 1.2 ppm (triplet) and δ 4.46 ppm (doublet). The signals at δ 1.2 ppm and δ 4.46 ppm result from β-anomeric protons and the methyl proton of EtG, respectively. The doublet at δ 4.46 ppm is relatively broad because of the rolling baseline and the inconsistent effect of water suppression.[15]

Nine spectra (from 0 h to 29 h) of the 4 volunteers were stacked (Fig. 2). Immediately after alcohol consumption (labeled as "2 h" spectrum), the ethanol resonance signals were much more intense than other signals, whereas EtG signals were not apparent. Ethanol resonances appeared at δ 1.11 ppm (triplet) and δ 3.56 ppm (quartet). Ethanol peaks remained strong for nine hour then rapidly decreased. At around 13 h, the ethanol peaks completely disappeared. Ethanol is rapidly metabolized to acetaldehyde and acetate by oxidation and acetate is excreted, so ethanol peaks were no longer detected.

Meanwhile, the EtG signal grew stronger over time and reached a maximum at 6-9 h, depending on the volunteer. This result is similar to measurements previously obtained from mass spectroscopy (data not shown), which indicated that the largest EtG concentration appeared at 5-7 h 30 min. Any small difference is the result of the different measuring mechanism between LC-MS and NMR. However, the trends of EtG concentration changes are well-correlated between methods. After EtG resonance reached its maximum value, the peak decreased gradually and disappeared completely between 20-25 h. We thus verified that EtG resonance is detectable by NMR up to 19 h after alcohol elimination. Weinmann *et al.* (2004) confirmed by mass spectroscopy that after 9 g of ethanol intake, EtG reached a maximum concentration at 8-10 h and is detectable over 30 h.[20] Furthermore, Halter *et al.* (2008) confirmed by LC-MS/MS

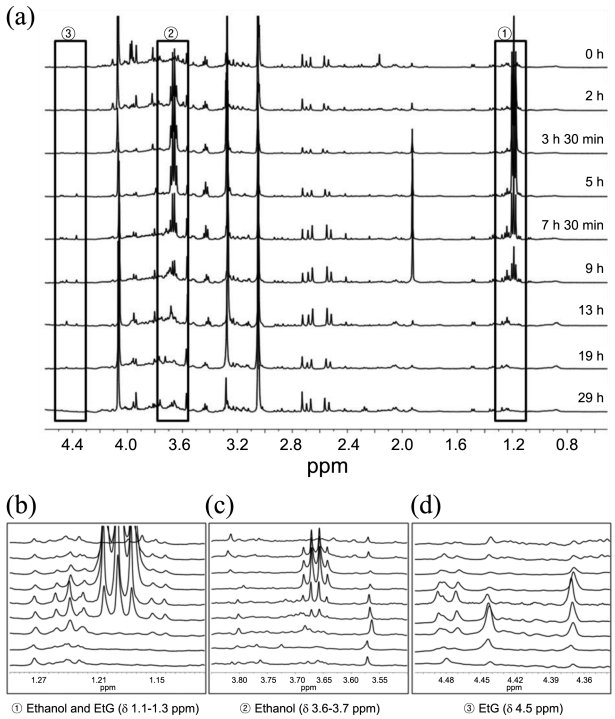

Figure 2. All data were normalized to the region of δ 0.5-4.5 ppm by Mnova 5.3.1. Normalized spectra were stacked to compare each EtG and ethanol concentration. Figure (a) shows the changing of NMR spectra of samples from volunteer #4 over times. (Indicated times on the spectra are the sample collection time. For example, the "0 h" spectrum is from a sample collected before alcohol consumption.) Ethanol passes through rapid oxidative metabolism; therefore, acetate, which is the oxidative metabolite observed at δ 1.9 ppm, increases with the elimination of ethanol. EtG is formed via non-oxidative ethanol metabolism. EtG signals at δ 1.2 ppm increased whereas ethanol signals (δ 1.1 ppm and δ 3.6 ppm) decreased. Three boxes describe the variation of ethanol and EtG signals and are expanded to (b), (c), (d), respectively. Meanwhile, the tendency of the singlet peak positioned at δ 4.35 ppm is similar to that of EtG, which could not assign with conventional urine library.

that the maximum EtG concentration occurs 8 h after 0.5-0.78 g/kg ethanol intake and remained detectable up to 24 h.[21]

Comparing all spectra over time, it is apparent that EtG signals increased with the elimination of ethanol signals. This trend was apparent in all data sets although the time courses of EtG and ethanol resonances differ slightly between subjects. We believe these minor variations are caused by factors including weight, the digestive power of each volunteer, and other individual differences.

A peak positioned at δ 4.35 ppm is not assigned. We took note of this peak because its variation tended to be similar to that of the EtG signal (Fig. 2(c)).

We verified that various metabolites were altered by alcohol intake. Acetate and acetaldehyde increased after alcohol consumption because these compounds are oxidative metabolites of ethanol. We also verified a high intensity acetate peak (δ 1.9 ppm). Acetate is generally detected in urine, but its intensity in 1 subject increased between 2 and 7 h after alcohol intake. However, we did not detect acetaldehyde. Moreover, alanine and lactate declined with time and 3-hydroxybutyrate increased. These results correspond to those of a previous study from Nicholas (2008)[11] and is caused by the effects of ethanol on gluconeogenesis.

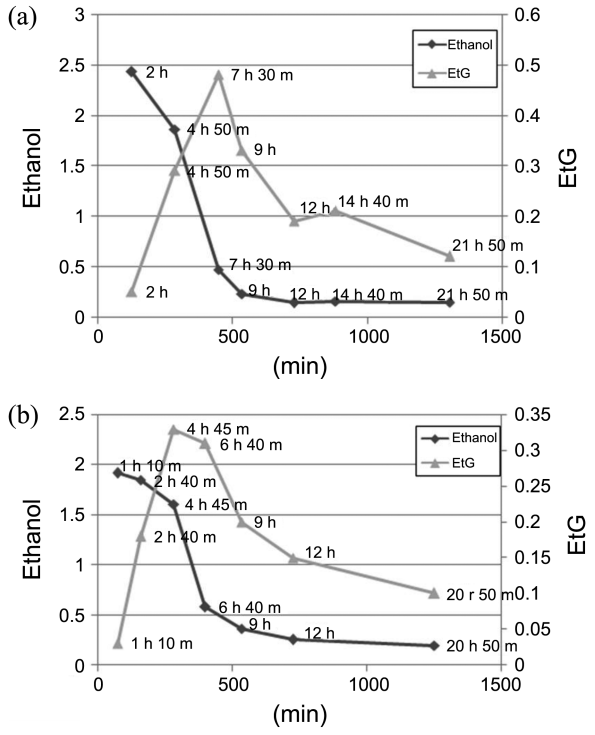

Figure 3. Variation of ethanol and EtG concentration with time. The value of intensity is normalized to TSP-d_4 concentration. In case of volunteer #3 (a), ethanol signal is decreased quickly and after the 9 h, the concentration of ethanol is less than 10% compared to the maximum. EtG is increased up to 7 h 30 min and decreased gradually. In case of volunteer #5 (b), like volunteer #3, ethanol is decreased dramatically and EtG is increased up to 6 h 40 min with similar way to volunteer #3. There are differences of time and integral values between two volunteers because of their dietary activity and health status.

Quantitative Analysis of EtG and Ethanol. In order to confirm the changes of EtG and ethanol concentration over time, the intensity for corresponding peaks was calculated. Might the total metabolic pool concentration is different each volunteers because of their individual dietary activity. Thus, these intensity values were normalized to TSP-d_4, the reference peak. For by monitoring of ethanol, δ 1.11 ppm signal (triplet) was selected because δ 3.56 ppm signal (quartet) was overlapped with other metabolite signals. In case of EtG, the δ 4.46 ppm signal (doublet) was not proper because of rolling baseline, thus δ 1.2 ppm signal (triplet) was used for quantitative analysis. Figure 3 shows that the variation of EtG and ethanol concentration of volunteer #3, and #5 with time dependency. Like as described in Figure 2, ethanol signal is decreased immediately after ethanol intake. At 9 h (volunteer #3) and 6 h 40 m (volunteer #5), the intensity of ethanol concentration is decreased to less than 10% compare to maximum concentration. And then, EtG is increased with time and decreased gradually up to about 21 h 50 m (volunteer #3) and 20 h (volunteer #5). There are differences of time and concentration of EtG between two volunteers because of their dietary activity and health status.

Metabolomic Discrimination Using Multivariate Analysis. In order to distinguish significant variation of 0 h samples (control sample) and the samples containing EtG, the useful pattern recognition method was utilized. For all sample, data were scaled using centered scaling prior to multivariate analysis. The binned each values of EtG signals were used to confirm whether EtG signals in NMR spectra are usable for determination of ethanol consumption. Multivariate analysis methods such as orthogonal projections to latent structures discriminant analysis (OPLS-DA) could be graphically showed as a score plot and provide powerful discrimination method.

Multivariate analysis was carried out on two groups with respect to sample collection time. First group includes 5 control and 5 ethanol intake samples which showed the largest differences in NMR spectra. The selected ethanol intake samples are 9 h 20 min (volunteer #1), 9 h (volunteer #2), 9 h (volunteer #3), 9 h 20 min (volunteer #4), and 9 h (volunteer #5). The other group contains control samples and about 20 h ethanol intake samples to examine the multivariate analysis method is available in low EtG concentration. The selected 5 ethanol intake samples are 20 h 20 min (volunteer #1), 24 h (volunteer #2), 21 h 40 min (volunteer #3), 19 h 40 min (volunteer #4), and 20 h 50 min (volunteer #5).

Figure 4 describes score plots of OPLS-DA results. These score plot show that control samples (labeled as "Control") are definitely separated from ethanol intake samples ("EtG") with principal component 1 (PC1). For first group, total 10 samples are distinctly categorized into two classes (Fig. 4(a)). Aforementioned, in this group, 5 ethanol intake samples are selected because these samples have the largest difference between ethanol and EtG concentration in NMR spectra. And the score plot shows clear classification of the control and ethanol intake group in PC1. For the other group,

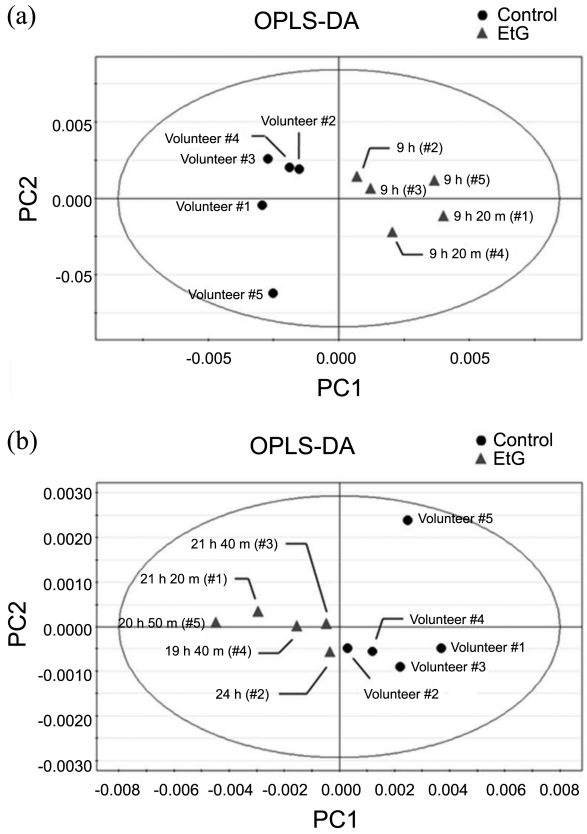

Figure 4. OPLS-DA score plot. Circle (●) indicates the control sample of each volunteer and triangle (▲) indicates the ethanol intake sample. (a) describes that the OPLS-DA score plot could demonstrate separation of the two groups into two major clusters in PC1. Control samples (normal person) are classified from 9 h samples clearly. And (b) also shows that control samples are separated from 20 h samples.

control samples are classified from ethanol intake samples, although the separation degree is lower than the former group by the decrease of the EtG concentration (Fig. 4(b)).

Discussion

In the field of forensics, it is important to be able to prove alcohol intake or to identify abusers. Ethanol is metabolized to acetaldehyde and acetate through oxidative metabolism. These oxidative metabolites, however, cannot be used as biomarkers because they are rapidly digested. Various other ethanol biological markers have been used, including CDT, GGT, AST, EtS, and EtG. Among them, EtG, the minor metabolite of ethanol, is particularly useful because of its high sensitivity and stability. The determination of EtG in the body is important in forensic chemistry, as it is used to demonstrate whether or not someone drank alcohol within the previous 12 h. EtG has been analyzed by mass spectroscopy, chromatography, enzyme-linked immunosorbent assay techniques.[22]

We used NMR to analyze ethanol intake. We collected urine samples from 5 healthy male volunteers over time before and after alcohol consumption, and analyzed the samples by NMR spectroscopy. We showed that EtG signals could be identified by NMR spectroscopy. EtG concentration increases after alcohol consumption and decreases slowly over time. Unlike the ethanol signal, the EtG signal is detectable up to 20-25 h after consumption. Acetate, which is an oxidative metabolite of ethanol, was also detected by NMR. Acetaldehyde, however, was not detected, likely due to low intensity of acetaldehyde peaks or the gap in data set collection times.

We also noted a singlet peak near δ 4.37 ppm, which behaved in a similar manner as the EtG signals. However, we could not identify the compound represented by this peak, which could be an unknown ethanol metabolite or a metabolite of another ingredient of the ethanol beverage. We used only one alcoholic beverage (So-ju) for our research. If this peak is related to a specific ingredient in the beverage we used, we suggest that similar additive peaks could be used to verify the type of alcohol a subject consumed. However, the origin of this specific peak must be determined by further study.

As a result of quantitative analysis, it is observed that the trend of variation between ethanol and EtG concentration is similar to that of NMR spectra. Moreover, multivariate analysis such as OPLS-DA shows that control samples are separated from ethanol intake samples even in the case of low EtG concentration.

In this study, we observed variations in EtG signals after ethanol intake by NMR spectroscopy. EtG signals could be detected up to 20 h after consumption. And we were able to monitor alcohol consumption without reference. These results lead to the conclusion that NMR spectroscopy without pretreatment can be used for the detection of alcohol abuse. Furthermore, multivariate analysis could be utilized to distinguish ethanol abusers from normal persons.

From these results, we expect that the combination of NMR measurement and multivariate analysis could be applied to the forensic field with similar ways as mass spectrometry combined liquid chromatography. Future studies would necessary to verify each material or chemical metabolites and establish concentration reference ranges over time.

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