Original article

Diagnostic meaning of urinary ethyl glucoside concentrations in relationship to alcoholic beverage consumption

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Abstract

Incidents and accidents often involve the drinking of alcoholic beverages. We investigated compounds that indicate the consumption of alcoholic beverages even after ethanol (EtOH) becomes undetectable in blood and urine. Ethyl glucoside (EG) has been isolated as a possible drinking marker, and a GC-MS/MS method for EG isomers has been developed. EG isomers in several alcoholic beverages were analyzed. In *sake*, only αEG was observed in high concentrations. In wine and beer, both α and βEG were detected. Whisky, however, did not contain EG. EtOH and EG concentrations were analyzed in urine up to 48 hours after ingestion. Maximum EtOH concentrations were reached in 1-2 hours and was mostly eliminated in 6 hours. Maximum EG concentrations were reached in 3-6 hours, gradually decreased, and remained low after 24 hours. After drinking *sake*, the αEG concentrations were much higher than that of other alcoholic beverages. After drinking wine or beer, βEG was detected, but lower than αEG. Also, αEG was detected in urine after drinking whisky that contained no EG. This suggested that αEG may be synthesized in vivo. Disaccharide-degrading enzymes such as α-glucosidase are present in the human small intestine. It was considered that αEG was synthesized when alcohol was consumed with certain foods, such as carbohydrates. In actual forensic autopsy cases, EtOH and EG isomer analysis provided useful information regarding drinking history. In conclusion, it is considered that urinary EG isomers can be used as drinking markers that complement EtOH analysis.

Keywords: urinary ethyl glucosides isomers, drinking marker, ethanol, alcoholic beverages, forensic autopsy cases

1. Introduction

Drinking alcohol is often involved in various incidents and accidents such as drunk driving and sex crimes. Blood and urinary ethanol (EtOH) detection and their concentrations are important for proof of drinking and evaluation of the degree of drunkenness. However, more than a few hours after drinking alcohol, EtOH cannot be detected in blood or urine.

Therefore, we have been searching for new compounds that could complement EtOH analysis, which can prove drinking even after EtOH is excreted and cannot be detected in the blood or urine. During routine forensic autopsy drug screening by gas chromatography-mass spectrometry (GC-MS), we occasionally experienced the detection of a prominent peak for ethyl glucoside (EG). When we researched the activity of these individuals just prior to death, it was suspected that the presence of EG could be related to alcohol consumption.

EG was identified by Japanese researchers as a constituent of *sake*, a Japanese alcoholic beverage brewed from rice [1, 2]. The EG detected from *sake* was the α isomer, α-ethyl glucoside [1]. The presence of EG in wine has been known, but was reported to be the β isomer, β-ethyl glucoside, in recent research [3, 4]. This research suggests that *sake* and wine contain different EG isomers. After analyzing several other alcoholic beverages, it was determined that some contained a mixture of EG isomers or no EG at all. These differences could provide useful information in the investigation of incidents

and accidents. Furthermore, as a result of carrying out a drinking experiment in volunteers, it was observed that EG could be detected in the urine for long periods after drinking [5].

The detection of EG during routine screening was accomplished in both blood and urine; however, this study focuses on the analysis of EG in urine only. There were several reasons for this. Firstly, urine is generally considered easier to collect and analyze than blood. For sample collection for a drinking study with volunteers, urine was chosen because it was easier and less invasive to collect urine than blood in such short time periods. Also, it was important to maximize the amount of time after consumption of alcohol that EG could be detected. Thus, urine was chosen as the matrix for the analysis of EG after drinking.

In this study, we investigated whether EG isomers could be used as drinking markers in urine after EtOH had disappeared from the body.

2. Materials and methods

2.1. The separation of ethyl glucoside isomers by gas chromatography-tandem mass spectrometry

The analysis methods for EG and EtOH were based on previously published reports [6, 9]. The analysis method for EG was validated using internationally accepted guidelines. The limit of detection (LOD) for both EG isomers was determined to be 0.3125 μg/mL in urine. The LOD for EtOH in urine was 0.01 mg/mL. The upper limits

of quantitation (ULOQ) for EG and EtOH in urine were 50 μg/mL and 5.12 mg/mL, respectively. The upper limits of detection (ULOD) for these assays were not examined. If any case quantitated over the ULOQ for either assay, the sample was diluted accordingly and re-analyzed.

2.1.1. Reagents and solvents

Reference standards of α-ethyl glucoside (98%) and β-ethyl glucoside (98%) were purchased from Wako (Osaka, Japan) and Combi-Blocks (San Diego, USA), respectively. β-octyl glucoside (>98%) for use as the internal standard (IS) was purchased from Dojindo Laboratories (Kumamoto, Japan). Solid-phase extraction cartridges (InertSep PSA, 500 mg/3 mL) were purchased through GL Sciences (Tokyo, Japan). Water was deionized using a Pure Water Purifier (RFP841AA) from Advantec (Chiba, Japan). Urease from the Jack bean, acetic anhydride, pyridine, and acetic acid were all purchased from Wako (Osaka, Japan). Acetonitrile was purchased from Sigma-Aldrich (Tokyo, Japan).

2.1.2. Sample preparation for urine

A 0.2 mL aliquot of urine, 0.2 mL of deionized water, and 0.05 mL of IS solution (1 mg/mL in methanol) were added to a 5-mL plastic tube. In order to remove urea, a major organic constituent of urine, 0.05 mL of a urease solution (3 mg/mL) was added to the tube, the mixture vortexed well and allowed to sit at room temperature for 30 min. A 2

mL aliquot of acetonitrile and 0.03 mL of acetic acid were added, vortex-mixed for 20 s, and centrifuged at 3,000 rpm for 5 min. The supernatant was passed through a Primary Secondary Amine (PSA) cartridge by gravity. The PSA cartridge was conditioned with 2 mL of acetonitrile prior to adding the sample. The eluate was evaporated to dryness under a nitrogen stream (75 °C) and microwaved at 500 W for 1.5 min to drive off any remaining moisture. To the dried residue, 0.2 mL of acetic anhydride and 0.01 mL of pyridine were added and mixed well, then microwaved at 500 W for 3.5 min for acetylation. After centrifugation, 0.15 mL was transferred to an autosampler vial and analyzed on the GC-MS/MS.

2.1.3. GC-MS/MS instrumentation and chromatographic conditions

The GC-MS/MS system was a GCMS-TQ8030 (Shimadzu, Kyoto, Japan). The columns used were two serially connected capillary columns (tandem columns) consisting of a Rtx-200 column (2 m × 0.18 mm i.d., 0.4 μm film thickness; Restek, USA) as the pre-column coupled to a Rtx-200 column (10 m × 0.15 mm i.d. × 0.25 μm) as the separation column connected by a SilTite® μ-Union connector (SGE) [7,8]. Electron ionization (EI) was employed at a voltage of 70 eV. The carrier gas was helium delivered at an initial pressure of 268.5 kPa and a constant flow of 2.00 mL/min. The oven temperature program was initially 70 °C for 0.5 min, ramped to 180 °C at 55 °C/min, held for 3 min, increased to 200 °C at 20 °C/min, held for 0.5 min, increased to 300 °C at

50 °C/min, and held for 3 min. The inlet, interface, and ion source temperatures were 250 °C, 280 °C, and 230 °C, respectively. The injection was made in split mode with a split ratio of 1:26. Split mode was employed to improve peak shape and allow for better separation of the EG isomers. The injection volume was one microliter and the total run time was 12 min. Initially, mass spectral data were collected using full scan and selected ion monitoring (SIM) modes simultaneously. The full scan mode targeted fragments in the range of *m/z* 40 to 550 at 5,000 amu/s. Due to the structural similarity of the target compounds, many of the same ion fragments were present in their scan spectra. Five ion fragments common to the target compounds were selected for monitoring in the SIM mode: *m/z* 81, 112, 141, 157, and 200. For targeting by MS/MS, detection was made in the multiple reaction monitoring (MRM) mode. Shooting standards for all relevant compounds were made in methanol and repeatedly injected into the GC-MS/MS to determine the optimal transitions and collision energies for each compound. The optimized MRM transitions, collision energies, and dwell times for αEG, βEG, and β-octyl glucoside as the IS can be seen in Table 1.

2.2. The concentration of ethyl glucoside isomers in alcoholic beverages

2.2.1. Alcoholic beverages

Japanese *sake* (7 brands), red wine (8 brands), white wine (6 brands), beer (7 brands), and whisky (7 brands) were purchased from local vendors.

2.2.2. Sample preparation for alcoholic beverages

In the sample preparation for *sake*, a 0.01 mL aliquot was diluted with deionized water to make 0.4 mL; for all other alcoholic beverages, 0.1 mL was diluted with deionized water to make 0.4 mL. The diluted alcoholic beverages were prepared according to the above sample preparation method, except the urease treatment was omitted [6].

2.3. Urinary ethanol and EG isomer concentrations after drinking alcoholic beverages

Three healthy adult volunteers consumed a sample of an alcoholic beverage containing about 0.5 g/kg body weight (30 to 50 g) of EtOH with a meal. The entire sample was to be consumed within 30 minutes. All experiments were performed after 3 days of abstinence from alcohol. Each participant consumed different brands of *sake*, red wine, white wine, beer, and whisky, for a total of 15 experiments (5 for each participant). Each volunteer also participated in a control experiment, where a meal was consumed with no alcohol.

A urine sample was collected before drinking and at 1, 2, 3, 4, 5, 6, 12, 24, 36, and 48 hours after the alcoholic beverage was completely consumed, for a total of 11 samples. The only restriction on the diet of the volunteers during the experiment was that they were to not eat or drink anything else containing ethanol until after the collection of the 48-

hour urine sample.

The concentrations of EtOH were analyzed by gas chromatography with flame ionization detection (GC-FID) [9]. EG isomers were measured by the method described in 2.1.

2.4. In vitro EG synthesis

It was investigated whether αEG could be synthesized by allowing α-glucosidase to act on disaccharides and ethanol as substrates [10].

About 0.1 g of Rat Intestine Acetone Powder (RIAP) was weighed into a 5-mL plastic tube. A 1 mL volume of 0.9% NaCl solution, 0.5 mL of 0.2M phosphate buffer solution (0.12 g of NaH_2PO_4 in 5 mL of deionized water, pH adjusted to 6-6.5 with 1N NaOH), 0.5 mL of deionized water, and 0.05 mL of an alcohol (ethanol, glycerol, or erythritol) were added to the tube. A 0.035 g sample of a disaccharide (lactose, sucrose, or maltose) was added, the mixture vortexed well and incubated at 37 ℃ for 2 hours. After incubation, the tube was centrifuged at 3,000 rpm for 5 min. A 0.2 mL volume of the supernatant was transferred to a separate 5-mL plastic tube and the sample prepared using the same method described in 2.1. Each experiment was repeated 3 times (n = 3) to confirm consistent results.

A summary of the results of enzymatic reactions using RIAP with various combinations of alcohols and disaccharides can be seen in Table 2.

2.5. Urinary EtOH and EG isomer concentration in forensic autopsy cases

A total of 320 forensic autopsies were conducted at Fukuoka University between January 2018 and December 2020. Of these, urine could be collected in 187 cases. A total of 53 cases were targeted in which the EG concentration exceeded the lower limit of detection in urine (0.5 μg/mL) by our routine toxicological screening method.

EtOH was analyzed by GC-FID [9]. The EG isomers were measured by the method described in 2.1.

The urinary EtOH and EG isomer concentrations were examined for all 53 cases. Of these cases, information about the drinking history immediately prior to death was known in about 1/3 of them. From these, 4 cases that represented a good mix of circumstances were selected that exemplified the relationship between the EtOH and EG concentrations elucidated by the drinking study.

2.6. Statistical analysis

Correlation, intergroup comparison, and reference statistics were analyzed by BellCurve for Excel (Social Survey Research Information Co., Ltd.). The Mann-Whitney U test was used for group comparison. Significant differences were assessed at $p < 0.05$.

3. Results

3.1. EG isomers in alcoholic beverages

The EtOH and EG isomer concentrations from various alcoholic beverages were shown in Table 3.

Wine, beer, and *sake* contained EG, but only one of the seven whisky brands contained a small amount of EG.

In *sake*, only the α isomer was observed, and βEG was not observed. Wine and beer contained both α and β isomers. The concentration of αEG in *sake* was more than 10 times higher ($p < 0.05$) than any of the other alcoholic beverages.

In wine, the concentration of βEG was significantly higher than that of αEG. When comparing red wine and white wine, the αEG concentration did not change, but the βEG concentration was significantly higher in red wine. Beer had significantly less βEG than αEG ($p < 0.05$).

3.2. Changes in urinary EtOH and EG isomer concentrations over time

Three healthy adult volunteers (No.1, No.2, and No.3) drank *sake*, beer, red wine, white wine, and whisky. Volunteer No.1 was a male in his 60s, No.2 was a male in his 40s, and No.3 was a female in her 30s. EtOH, αEG, and βEG concentrations were measured from urine sampled at various intervals from before drinking to 48 hours after complete consumption of a sample of an alcoholic beverage. The results of the drinking study are

summarized in Table 4.

In every experiment, the urinary EtOH concentration reached its maximum 1 to 2 hours after drinking, and was mostly eliminated by 6 hours after drinking.

The urinary αEG concentration reached the maximum concentration 3-6 hours after drinking, gradually decreased and remained low after 24 hours, although there were slight differences depending on the alcoholic beverage and the volunteer. The maximum αEG concentration in the urine of *sake* drinkers ranged from 744 to 2,275 μg/mL, and was much higher than that of the other alcoholic beverages.

Fig. 1 shows a scatter plot that represents the relationship between EtOH and αEG concentrations over time after the consumption of a sample of an alcoholic beverage. Since the relationship between EtOH and αEG produced the same pattern regardless of the volunteer or the alcoholic beverage consumed, all of the data was combined and represented in this single plot. The points along the plot were determined by taking the median value of EtOH and αEG from all 15 drinking experiments at each time interval (Table 4). The red points connected with the solid red line represents the αEG concentration over time, while the blue dots and line represents the EtOH concentration over time. The whiskers on each dot represent the upper and lower quartile of the median concentration. Since αEG is produced in vivo from the consumption of certain foods, a cut-off value was established that represents the highest value of EG that is not due to the consumption of ethanol. This value was decided by examining 48 urine samples collected

from the 3 volunteers during a period when they were not consuming alcoholic beverages. The median of these αEG concentrations (n = 48) was 4.85 μg/mL. The 75th percentile (upper quartile) of this value was 15.2 μg/mL. This was converted to a % of αEG Cmax by dividing each calculated αEG Cmax from the drinking experiments (n=15) by 15.2 and then taking the median of those values. The dotted red horizontal line displayed in Fig. 1 represents the "cut-off" for αEG. The dotted blue horizontal line is the "cut-off" for EtOH and was set to 0.1 mg/mL (as a percent of EtOH Cmax) because 0.1 mg/mL is often used by laboratories as the lower limit of quantitation to indicate the consumption of alcohol. Displaying these together on the Fig. 1 scatter plot visually represents the relationship between the concentrations of EtOH and αEG over time and can predict the approximate time after drinking by the location on the plot based on the measurement of EtOH and αEG concentrations. These cut-off values showed 3 clear "zones" that may help identify the drinking behavior prior to collection of the urine sample. Zone 1, the "Over-Over Zone", represents EtOH and αEG concentrations that are both over the established cut off values. These are likely cases where the alcoholic beverage was consumed within 0 to 4 hours. Zone 2, the "Under-Over Zone", is the zone where the EtOH concentration is under the cut-off, but the αEG concentration is over the cut-off. These cases fall within 4 and 26 hours after consumption of the alcoholic beverage. Finally, Zone 3, the "Under-Under Zone", depicts the time EtOH and αEG concentrations have both fallen below their respective cut-off values. This could represent greater than

26 hours after consumption of the alcoholic beverage, or no consumption of any alcoholic beverage at all.

The βEG concentrations were much lower than the αEG concentrations in urine. Since the contribution of βEG was negligible, it was not included in the scatter plot. In *sake* and whisky, βEG could hardly be detected in all three volunteers. In wine and beer, βEG was detected, and its change over time was in good agreement with the change in αEG (data not shown).

In the control experiments, a slight elevation of EG was sporadically observed.

3.3. Synthesis of αEG in vitro

When EtOH and the disaccharides maltose or sucrose were reacted with RIAP, it was confirmed that αEG and glucose were produced. The proposed biochemical reaction between maltose and ethanol and the resultant GC-MS/MS chromatogram was shown in Fig. 2.

3.4. Urinary EtOH and EG isomer concentrations in forensic autopsy cases

The sex, age, and postmortem interval (PMI) of the 53 cases that were examined were summarized in Tables 5 and 6.

EtOH and EG isomer concentrations from the 53 cases were summarized in Table 7.

The median concentration of total EG was 30.8 μg/mL, and the 75th percentile (upper quartile) was 70.7 μg/mL (minimum value 1.39 μg/mL, maximum value 863 μg/mL).

These concentrations did not show any correlation or significant difference with age or sex. However, the EG concentration showed a significant difference between 24 hours and 48 hours of PMI with the Mann-Whitney U test. The relationship between EtOH and EG concentration in urine was shown in the distribution map (Fig. 3) of 53 actual forensic autopsy cases. No clear correlation was found between EtOH concentration and age ($r = -0.180$), between EG concentration and age ($r = -0.123$), between EtOH concentration and αEG concentration ($r = 0.179$), or between αEG concentration and βEG concentration ($r = -0.039$).

Of the 53 cases where EG was detected in urine during routine screening, approximately 1/3 of them had information available about the drinking history of the deceased shortly before death. In order to demonstrate the potential usefulness of analyzing EG in postmortem autopsy cases, four forensic autopsy cases were selected where the drinking information immediately prior to death was known, and the resultant EtOH and EG concentrations exemplified the relationship between EtOH and EG concentrations over time that were elucidated from the drinking study. The concentrations of EtOH and EG isomers in the 4 cases are summarized in Table 8. These 4 cases are identified by red diamonds in Fig. 3.

Case 1: A female in her late 50s died of drowning. She drank "*shochu*" (Japanese distilled liquor) in the evening. Later she took a bath, and her husband, who was concerned after she did not come out of the bath for a long time, found her drowned in the bathtub. The

time of death after drinking alcohol was estimated to be a few hours.

Case 2: A male in his late teens died of acute alcohol intoxication. He had been drinking with his friends since midnight. He vomited several times during the night but fell asleep and was left alone. The next morning, he did not respond to being called and was cold to the touch, so his friends called an ambulance, but he had already died. At the time of death, about 3-6 hours had passed since drinking.

Case 3: A female in her late 20s died of drowning. The night before her death she drank at a party, stayed at a friend's house, and returned home around noon. She took a bath at home after dinner with her family. One hour after going into the bath the gas water heater alarm went off, and when the mother checked the bathroom, she found her daughter drowned in the bathtub. At the time of death, it was estimated that about 21 hours had passed since drinking.

Case 4: A male in his late 40s died of acute myocardial infarction. He drank beer the night before his death. He went to work the next morning at 6 am. At 6:40 am, a surveillance camera filmed him going into the bathroom. At 2:12 pm, he was found lying on the floor in the bathroom. The time of death after drinking alcohol was estimated as half a day.

4. Discussion

4.1. The concentration of ethyl glucoside isomers in various alcoholic beverages

Distilled liquors have a high alcohol content made by further distilling brewed liquor

and brewed dregs. Therefore, in distilled liquors, EG is removed in the process of distillation. One brand of whisky contained a very small amount of EG, but it is possible that it remained after the distillation process or was added after distillation. Basically, it is considered that distilled liquors do not contain EG.

Brewed liquor, however, is made from the fermentation of grains and fruits. The only secondary processing after fermentation is clarification and filtration. In brewed liquors, various compounds such as alcohol and sugar are produced in the fermentation process. *Sake* is manufactured from the starch in rice. Amylase, an enzyme found in rice malt, breaks down starch into maltose and glucose. EG is synthesized from α-glucosidase in rice malt using ethanol produced by maltose and yeast. As *sake* is made from starch with polymerized α-glucose, the α-isomer is produced [11, 12]. In previous studies, αEG was detected from *sake* [1, 3]. As a result of our research, it was confirmed that *sake* contains 10 times more EG than wine and beer, and that the EG in *sake* is exclusively αEG.

Wine, which is primarily made from grapes, is similar to other alcoholic beverages made from plants in that it contains many β-glucose derivatives derived from cellulose [13]. In this study, wine contained both α and βEG, similar to previous studies [3, 4]. Furthermore, the βEG concentration was significantly higher than αEG. It was also revealed that the βEG concentration was significantly higher in red wine than in white wine. Beer is made of barley which contains maltose and cellulose. It contained both α and βEG, and significantly less βEG than αEG. From the above results, it was considered

possible that the specific type of alcoholic beverage could be identified by measuring the concentrations and ratios of the EG isomers.

4.2. The change over time of urinary ethanol and EG isomer concentrations after drinking alcoholic beverages

In previous studies of EG plasma kinetics, both α and βEG were transported through the rat or hamster small intestinal wall [14-18], and rapidly absorbed into the blood stream, then easily excreted into urine [19]. Mishima et al. reported that a portion of α and βEG escaped metabolism and excretion [14, 20]. Approximately 80% of EG was recovered within 6 h after oral administration and small quantities were detected in urine in the 24-48 h period in the rat [15].

In another study, EG was detected by metabolomics analysis of human urine by Nuclear Magnetic Resonance, which was characteristic of human urine in Asians who were drinking an alcohol similar to *sake* [5]. As a result of a drinking experiment by volunteers, it was reported that EG was detected even 28 hours later after drinking [5]. In our study, EG was measured with EtOH until 48 hours, and αEG was confirmed in all samples.

The urinary αEG concentration after drinking *sake* was much higher than that of the other alcoholic beverages. Since the concentration of αEG in *sake* itself was over 10 times higher than that of the other alcoholic beverages, it was considered that the high

concentration of αEG in the urine after drinking *sake* reflects the fact that it was *sake* that was consumed.

βEG was clearly detected in urine after drinking wine or beer, and slightly detected in urine after drinking *sake* and whisky. The maximum concentration of βEG in urine after drinking white wine or beer was about the same. The maximum concentration of βEG in urine after drinking red wine was higher than that of white wine or beer. The concentration of βEG in urine might be a good indication that alcoholic beverages containing βEG have been consumed. However, although the concentration of βEG was significantly higher than αEG in wine, and α and βEG concentrations in beer were similar, in all the drinking experiments the concentration of αEG in the urine was significantly higher than that of βEG. Furthermore, αEG was detected in the urine after drinking a whisky brand that did not contain any αEG. It was considered that αEG may be produced in the body after drinking alcohol.

Therefore, we examined the relationship between EtOH and αEG concentrations in urine over time after drinking alcoholic beverages (Fig. 1). In the early stages after drinking, until about 4 hours, both EtOH and αEG exceed the cut-off values (EtOH > 0.1 mg/mL and αEG > 15.2 μg/mL). Because of EtOH's short half-life, the EtOH drops rapidly to below the cut-off value while the αEG concentration remains over the cut-off from about 4 to 26 hours after drinking. At 26 hours after drinking alcohol, both EtOH and αEG have been almost completely removed, and both concentrations are below the

cut-off values.

4.3. EG synthesis in vitro

The αEG contained in *sake* uses maltose and ethanol derived from the polysaccharide of α-glucose bond as substrates and is produced during *sake* brewing by the sugar transfer reaction of α-glucosidase [11]. Disaccharidases such as α-glucosidase are present in the human small intestine [21, 22]. Therefore, we considered that αEG may be produced if disaccharides such as maltose or sucrose, both disaccharides that contain glucose, are mixed with ethanol in the small intestine.

So, the possibility of αEG synthesis in vivo was investigated. When experiments were conducted using RIAP instead of human small intestine, it was confirmed that αEG and glucose were synthesized from the reactions of ethanol with the disaccharides maltose or sucrose. When EtOH and lactose were reacted with α-glucosidase, however, αEG was not produced.

This suggests that when certain disaccharides are present in the small intestine and EtOH is consumed, αEG is synthesized by the enzyme α-glucosidase in the small intestine. Since it was clarified that αEG is produced in vivo by eating a meal while drinking, the cause of the detection of αEG in the drinking experiment of whisky containing no EG was clarified. In addition, the reason why αEG was detected at higher concentrations than βEG in the urine of wine and beer drinkers, even though wine and beer contain less or the

about the same amount of αEG than βEG, was clarified.

4.4. Urinary EtOH and EG isomer concentrations in forensic autopsy cases

We investigated the relationship between urinary EtOH and EG concentration and the relationship between αEG and βEG in 53 actual forensic autopsy cases. A urinary total EG concentration over 30.8 μg/mL may be a reference value for suspected drinking. In the group with a PMI of greater than 24 hours but less than 48 hours, the EG concentrations were significantly higher than any other group, because there were many abnormally high EG cases in this group.

In case 1, a female who died within 2-3 hours of drinking "*shochu*", a Japanese distilled liquor, without any eating, the urinary EtOH concentration was very high, but the EG was not so high, and βEG was below our LOQ. In case 2, after heavy drinking with friends, a male died of acute alcohol intoxication in a short period, so it was probable that both the blood and urinary EtOH and EG levels reached high levels. In case 3, since 21 hours had passed since drinking alcohol, it was considered that EtOH had been completely eliminated from the urine and EG was still being excreted. In case 4, a male was considered to have died between 6 to 12 hours after drinking beer. Therefore, the EtOH concentration was below the LOQ, and EG could still be detected. In this case, the detection of βEG could corroborate the consumption of beer.

These cases in which information was obtained about recent drinking and eating

history were sufficient to demonstrate the effect of diet and alcohol consumption on urinary EG and EtOH concentrations. Thus, it was considered possible to estimate the status of drinking and food intake before death and the passage of time from the urinary EG and EtOH concentrations.

Ethyl glucuronide (EtG), a metabolite of ethanol, has been reported as an indicator of ethanol intake even after ethanol has disappeared from the blood [23-25]. EG, however, is not a metabolite of EtOH, but an ingredient of some alcoholic beverages, and is synthesized by the enzyme in the small intestine, α-glucosidase, in the living body. The Cmax in blood of EtG was reported to be 4 h after drinking and the half-life is 2.2 h. In urine, the Tmax is 4.75 h and can be detected for up to 30 hours [23]. However, since EtG is a metabolite of EtOH, there is no way to differentiate between what kind of alcoholic beverage was consumed. This could be important information to corroborate recent activity. Since EG can be detected in urine up to about 24 h, it was considered that it can be detected long after drinking alcohol, similar to EtG. Furthermore, the EG isomer concentrations could be useful to determine the specific alcoholic beverage that was consumed.

Even after EtOH is eliminated completely from the body, it is considered that recent drinking activity can be elucidated by analyzing EG together with EtG.

In conclusion, it is considered that urinary EG concentrations can provide useful information for estimating the presence or absence of drinking, the elapsed time after

drinking, and the specific alcoholic beverage that has been consumed. Furthermore, it is considered that urinary EG can be a drinking marker that complements EtOH or its metabolite, EtG.

Ethical approval

This study was conducted with the approval of the Fukuoka University Medical Ethics Committee (2017M067, 2018M056).

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Declaration of Competing Interest

The authors declare that there were no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Legends to Figures and Tables

Figures

Fig. 1 The relationship between EtOH and αEG concentrations over time

Blue solid line: plot of median concentration of EtOH

Red solid line: plot of median concentration of αEG

Blue dotted line: "cut-off" concentration of EtOH (0.100 mg/mL)

Red dotted line: "cut-off" concentration of αEG (15.2 μg/mL)

Pink area: "Over-Over Zone" – both EtOH and αEG over cut-off value

Yellow area: "Under-Over Zone" – EtOH under cut-off but αEG over cut-off

Blue area: "Under-Under Zone" – Both EtOH and αEG under cut-off

Fig. 2 Biochemical reaction between maltose and ethanol in vitro

a: Proposed chemical reaction for the synthesis of α-Ethyl Glucoside

b: GC-MS/MS chromatogram of the chemical reaction product

Fig. 3 Distribution map of urinary EtOH and total EG concentrations in forensic autopsy cases

Tables

Table 1 GC–MS/MS conditions (quantitation ions in bold)

Table 2 Enzymatic reactions using RIAP with various combinations of alcohols and disaccharides

Bold denotes the production of α-ethyl glucoside (or its deuterated form) and glucose.

Each reaction was repeated multiple times (n = 3) to confirm consistent results.

Table 3 Ethanol and ethyl glucoside isomer concentrations in commercially available alcoholic beverages

ND = Not Detected

Table 4 EtOH and EG concentrations over time in various alcoholic beverage drinking experiments

ND: not detected NC: urine not collected

*There was no consumption of alcohol from 48 hours before to 48 hours after the collection of urine began

Table 5 Summary of age and sex in forensic autopsy cases

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Table 6 Summary of postmortem interval in forensic autopsy cases

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Table 7 EtOH and EG concentrations in forensic autopsy cases

Table 8 Concentrations of EtOH and EG in 4 cases

LOQ = Limit of quantitation

Fig. 1 The relationship between EtOH and aEG concentrations over time

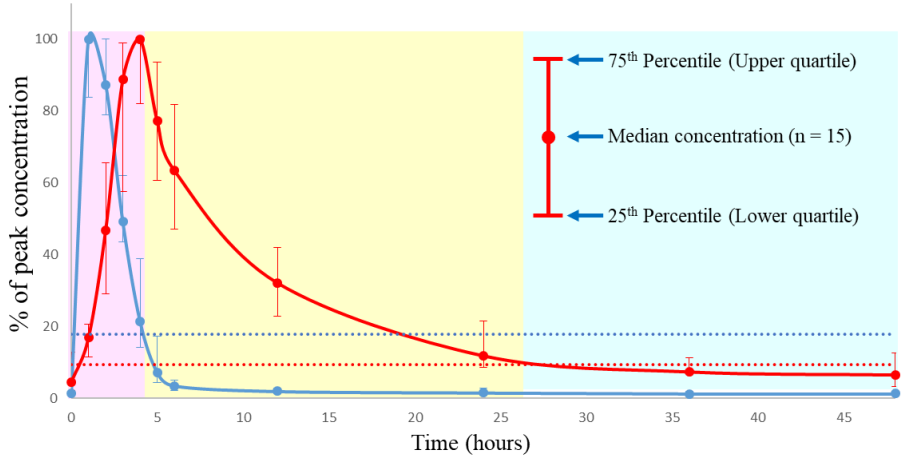
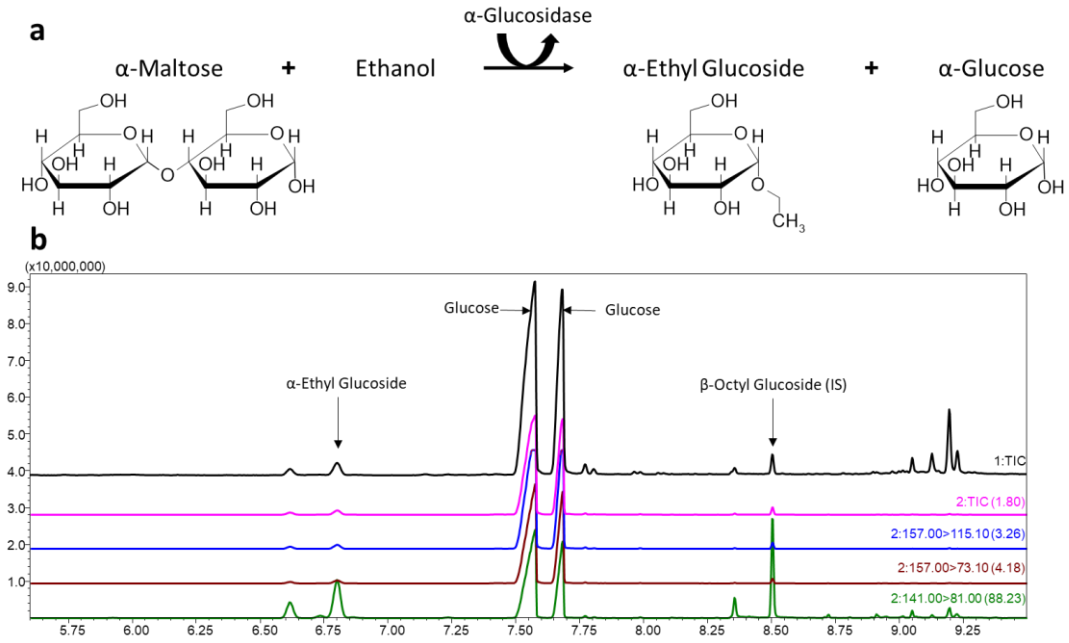


Fig. 2 Biochemical reaction between maltose and ethanol in vitro



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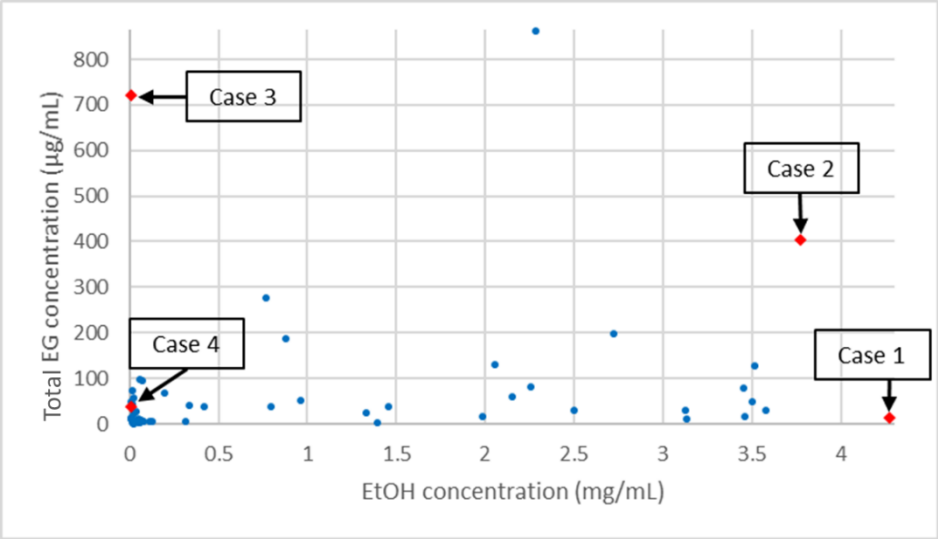

Table 1 GC–MS/MS conditions (quantitation ions in bold)

Retention Time (min)	Compound	Transitions (*m/z*)	Collision energy (eV)	Dwell time (ms)
7.055	α-Ethyl glucoside	**157.0 > 115.1**	6	30
		157.0 > 73.1	12	30
		141.0 > 81.0	6	30
7.153	β-Ethyl glucoside	**157.0 > 115.1**	6	30
		157.0 > 73.1	12	30
		141.0 > 81.0	6	30
8.559	β-Octyl glucoside (IS)	**157.1 > 115.1**	3	30
		157.0 >73.1	12	30
		141.0 > 81.0	9	30

Table 2 Enzymatic reactions using RIAP with various combinations of alcohols and disaccharides

Substrates		Enzyme	Products		
Alcohol	Disaccharide		Glucoside	Monosaccharide	
Ethanol	**Maltose**	Rat Intestine Acetone powder	**α-Ethyl glucoside**	**Glucose**	
Ethanol-d6	**Maltose**		**α-Ethyl-d5 glucoside**	**Glucose**	
Ethanol	**Sucrose**		**α-Ethyl glucoside**	**Glucose**	Fructose
Ethanol	Lactose		β-Ethyl galactoside	Glucose	β-Galactose
Glycerol	Maltose		α-Glucosylglycerol	Glucose	
Glycerol	Sucrose		α-Glucosylglycerol	Glucose	Fructose
Erythritol	Maltose		α-Glucosylerythriol	Glucose	
Bold denotes the production of α-ethyl glucoside (or its deuterated form) and glucose.					
Each reaction was repeated multiple times (n = 3) to confirm consistent results.					

Table 3 Ethanol and ethyl glucoside isomer concentrations in commercially available alcoholic beverages

Alcoholic Beverage	Red wine (n=8)			White wine (n=6)			Beer (n=7)			*Sake* (n=7)			Whisky (n=7)		
No.	EtOH (abv)	αEG (μg/mL)	βEG (μg/mL)	EtOH (abv)	αEG (μg/mL)	βEG (μg/mL)	EtOH (abv)	αEG (μg/mL)	βEG (μg/mL)	EtOH (abv)	αEG (μg/mL)	βEG (μg/mL)	EtOH (abv)	αEG (μg/mL)	βEG (μg/mL)
1	14.5	23.3	158	11	32.8	134	5	26.0	24.2	15~16	988	ND	40	ND	ND
2	12.5	29.4	62.0	11.5	32.8	51.6	5.5	32.2	23.4	16	2339	ND	40	17.7	24.9
3	12.5	27.0	71.1	12.5	26.0	38.3	4.5	27.5	21.2	14~15	1788	ND	37	ND	ND
4	13	24.1	124	13	20.6	56.7	5	20.8	17.8	15	2489	ND	37	ND	ND
5	12	25.8	78.5	12	19.6	49.6	4.5	48.5	20.0	13~14	1097	ND	40	ND	ND
6	13.5	35.4	147	12	27.7	57.6	5	47.7	23.0	15~16	1750	ND	40	ND	ND
7	13.5	36.3	130				5	33.4	21.5	14.5	1622	ND	7	ND	ND
8	14	49.6	168												
Average		31.4	117		26.6	64.7		33.7	21.6		1725	ND		ND	ND
Median		28.2	127		26.8	54.2		32.2	21.5		1750	ND		ND	ND
Max.		49.6	168		32.8	134		48.5	24.2		2489	ND		17.7	24.9
Min.		23.3	62.0		19.6	38.3		20.8	17.8		988	ND		ND	ND

ND = Not Detected

Table 4 EtOH and EG concentrations over time in various alcoholic beverage drinking experiments

Sake	15~16% abv			16% abv			16% abv		
	Volunteer No. 1			Volunteer No. 2			Volunteer No. 3		
Time after drinking (hours)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)
0	0.010	5.96	1.12	0.008	2.80	ND	0.004	5.74	ND
1	0.602	163	ND	0.811	569	ND	1.01	160	ND
2	0.532	744	ND	0.572	1554	ND	1.12	558	ND
3	0.457	397	ND	0.335	1273	ND	NC	NC	NC
4	0.334	445	ND	0.169	1874	ND	0.498	2271	4.64
5	0.167	438	ND	0.045	1790	ND	0.218	2105	4.44
6	0.057	523	ND	0.009	1123	ND	0.069	1647	2.37
12	0.022	310	ND	0.003	454	ND	0.014	575	ND
24	0.019	366	ND	0.005	143	ND	0.006	117	2.14
36	0.013	215	ND	0.009	88.1	ND	0.013	151	ND
48	0.018	62.1	ND	0.007	50.6	ND	0.011	22.9	2.06

Beer	6% abv			10% abv			5% abv		
	Volunteer No. 1			Volunteer No. 2			Volunteer No. 3		
Time after drinking (hours)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)
0	0.010	16.5	1.49	0.019	3.05	ND	0.006	3.42	ND
1	0.461	28.4	1.36	0.379	20.1	ND	0.557	13.6	ND
2	0.373	97.5	2.76	0.253	79.0	ND	0.481	48.2	2.11
3	0.281	105	3.18	0.085	113	1.48	0.263	137	5.41
4	0.132	164	4.50	0.021	53.6	0.943	0.123	155	4.99
5	0.048	202	4.38	0.000	17.8	ND	NC	NC	NC
6	0.021	123	3.04	0.000	20.7	ND	0.022	134	2.61
12	0.014	82.1	2.52	0.013	15.7	ND	0.011	74.1	ND
24	0.008	12.0	0.901	0.019	28.4	ND	0.009	26.2	1.37
36	0.009	15.1	0.882	0.020	17.7	ND	0.008	20.3	0.860
48	0.008	6.86	10.4	0.021	10.1	ND	0.010	18.3	0.915

Red wine	13% abv			13% abv			14% abv		
	Volunteer No. 1			Volunteer No. 2			Volunteer No. 3		
Time after drinking (hours)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)
0	0.008	36.0	1.67	0.004	3.26	1.64	0.007	22.5	2.76
1	0.498	50.3	1.46	0.260	11.8	1.04	1.05	26.3	1.76
2	0.718	149	5.52	0.223	56.5	ND	1.56	51.9	13.2
3	0.501	217	12.6	0.110	72.4	3.45	0.731	175	76.3
4	0.336	243	19.3	0.020	60.5	3.84	0.137	103	41.3
5	0.165	229	20.1	0.011	37.0	ND	0.304	116	61.3
6	0.039	188	20.1	0.010	15.4	ND	0.021	62.0	25.3
12	0.014	95.1	16.4	0.004	15.8	ND	0.007	39.8	10.0
24	0.011	25.5	2.88	0.010	9.89	ND	0.014	18.0	4.75
36	0.007	17.6	2.53	0.005	5.79	ND	0.004	4.67	1.40
48	0.006	7.08	1.62	0.004	8.93	ND	0.007	35.1	ND

White wine	13 % abv			12% abv			12% abv		
	Volunteer No. 1			Volunteer No. 2			Volunteer No. 3		
Time after drinking (hours)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)
0	0.010	3.69	ND	0.006	1.86	0.976	0.019	4.85	ND
1	0.469	28.3	ND	0.520	1.23	ND	0.425	8.24	ND
2	0.507	73.2	ND	0.399	10.5	1.72	0.323	18.9	2.30
3	0.353	126	2.86	0.176	38.2	6.14	0.227	37.3	4.96
4	0.153	161	3.09	0.082	39.9	5.35	0.053	30.9	4.39
5	0.057	155	3.32	0.023	22.2	2.72	0.016	27.2	3.52
6	0.033	140	2.89	0.015	8.75	1.19	0.013	24.5	2.52
12	0.013	67.9	1.82	0.013	9.09	1.05	0.012	21.8	1.59
24	0.005	15.5	0.921	0.009	7.23	1.09	0.014	14.3	ND
36	0.012	15.6	1.11	0.008	3.38	ND	0.000	2.71	ND
48	0.010	7.51	ND	0.013	6.24	ND	0.003	4.74	1.64

Whisky	40% abv			40% abv			7% abv		
	Volunteer No. 1			Volunteer No. 2			Volunteer No. 3		
Time after drinking (hours)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)
0	0.007	11.0	1.56	0.017	15.2	1.69	0.010	49.1	1.56
1	0.747	15.8	1.05	0.267	19.0	1.45	0.523	19.5	0.849
2	0.745	73.2	ND	0.519	22.7	1.35	0.674	30.2	1.05
3	0.466	116	ND	0.255	80.8	2.55	0.323	57.3	1.47
4	0.366	230	ND	0.085	91.2	3.70	0.226	101	1.98
5	0.054	192	ND	0.013	70.4	2.50	0.039	95.7	1.48
6	0.025	222	ND	0.008	53.5	1.99	0.020	106	1.82
12	0.009	99.6	ND	0.004	17.1	ND	0.017	38.2	0.792
24	0.006	30.7	ND	0.005	5.93	ND	0.017	33.0	1.59
36	0.007	17.0	1.76	0.005	5.45	ND	0.015	19.0	1.03
48	0.006	11.1	ND	0.000	2.88	ND	0.014	14.0	1.38

Control*									
	Volunteer No. 1			Volunteer No. 2			Volunteer No. 3		
Time after drinking (hours)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)
0	0.005	3.41	2.05	ND	2.48	1.03	0.017	47.6	15.2
1	0.003	2.65	1.93	ND	2.09	0.747	0.017	37.5	7.19
2	NC	NC	NC	ND	1.61	0.704	0.018	35.8	7.07
3	0.005	3.68	2.03	0.004	1.63	0.836	0.015	19.8	4.63
4	ND	4.11	1.81	ND	0.909	0.754	NC	NC	NC
5	0.004	4.92	2.03	ND	0.871	0.721	NC	NC	NC
6	ND	6.51	2.37	ND	0.506	0.607	0.033	7.26	2.63
12	ND	4.60	1.53	ND	0.639	0.690	ND	9.55	5.54
24	0.005	115	31.4	ND	0.874	0.973	ND	8.75	1.63
36	0.003	31.5	5.44	ND	1.79	0.826	0.016	5.87	1.76
48	0.012	15.4	4.30	ND	4.11	0.808	ND	9.06	2.02

Table 5 Summary of age and sex in forensic autopsy cases

			Age	
	Group	n	Mean	SD
Sex	Male	45	52.0	18.7
	Female	8	56.8	19.4
	Total	53	52.7	18.8
SD = Standard Deviation				

Table 6 Summary of postmortem interval in forensic autopsy cases

			EG (μg/mL)		
	Group	n	Median	Max.	Min.
PMI	≦ 24 hours	10	12.4	49.6	1.39
	24 - 48 hours	31	47.9	863	1.57
	48 hours - 10 days	8	11.0	67.1	4.77
	10 days <	4	22.4	81.8	4.72
PMI = postmortem interval					

Table 7 EtOH and EG concentrations in forensic autopsy cases

	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)
Mean	1.09	74.4	7.95
Standard Deviation	1.35	159	12.8
Median (50%)	0.199	27.5	2.87
Upper Quartile (75%)	2.20	54.4	5.70
Lower Quartile (25%)	0.024	7.25	1.86
Maximum	4.28	863	53.7
Minimum	0.005	0.548	0.844

Table 8 Concentrations of EtOH and EG in 4 cases

Case No.	EtOH (mg/mL)	αEG (μg/mL)	βEG (μg/mL)
1	4.28	12.0	<LOQ
2	3.78	404	<LOQ
3	<LOQ	716	2.48
4	<LOQ	36.3	1.67
LOQ = Limit of quantitation			