Levels of Ethyl Glucuronide and Ethyl Sulfate in Oral Fluid, Blood, and Urine After Use of Mouthwash and Ingestion of Nonalcoholic Wine

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Abstract

The aim of this study is to investigate the concentrations of ethyl glucuronide (EtG) in oral fluid and both EtG and ethyl sulfate (EtS) in blood and urine following intense use of mouthwash and ingestion of nonalcoholic wine, which are proven to contain 3 mg/L EtG, 1.5 mg/L EtS, and 0.2 g/L ethanol. Twelve subjects participated in a controlled experiment. All subjects ingesting nonalcoholic wine showed urine samples negative for EtG but positive for EtS (Cmax 2.15 mg/L). All four subjects using mouthwash were negative for EtG and EtS in urine. All samples of oral fluid were negative for EtG and all samples of blood were negative for EtG and EtS. This study showed that ingestion of EtG and EtS as components of nonalcoholic wine lead to detection of urine EtS only, suggesting superior bioavailability of orally ingested EtS compared to EtG. This possibility of false-positive EtS results in urine after ingestion of nonalcoholic wine is important to remember when using EtG and EtS as relapse markers for alcohol. Finally, the study showed that a positive EtG or EtS result after accidental alcohol exposure is unlikely in blood and oral fluid.

Introduction

In a situation like workplace drug testing, verification of alcohol intake could be desirable. Traditionally, this is done by measuring ethanol in blood, breath, or urine, but because ethanol is cleared rapidly from the body, only very recent intakes could be detected (1,2). Therefore, the non-oxidative ethanol metabolites ethyl glucuronide (EtG) and ethyl sulfate (EtS) are increasingly used for this purpose (3). Used as relapse markers, they are most frequently measured in urine, where they have a detection time of approximately 24 h after ingestion of a low dose of ethanol or up to five days after ingestion of large, repeated doses (4,5).

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Oral fluid has become an important alternative to urine as a matrix in drug testing programs (6,7), and we have recently suggested the use of this medium to test for alcohol ingestion by measurement of EtG. Firstly, the introduction of oral fluid makes the sampling procedures easier to perform and supervise compared to urine. Secondly, oral fluid reflects the presence of alcohol or drugs in blood better than urine (8–10). According to a recently performed study, this is also the situation for EtG (11).

One problem when using EtG and EtS as relapse markers for alcohol ingestion is the possibility of having too good of a sensitivity for some purposes, as EtG and EtS could also be detected after incidental ingestion of alcohol. Three papers have previously tried to assess this problem, indicating levels of EtG in urine of about 0.1–0.3 mg/L after intense exposure to mouthwash or hand sanitizers (12–14). To the authors' knowledge, no such published data exist for EtG or EtS measured in blood or oral fluid. In addition, it is theoretically possible that direct ingestion of EtG and EtS in the absence of ethanol, a situation possible in nonalcoholic wines, could yield positive EtG and EtS results, which leads to a false assumption of alcohol ingestion. This question has, to the authors' knowledge, not so far been addressed in the literature.

The aim of this study is to investigate the possibility of such "false-positive" EtG results in oral fluid, and both EtG and EtS results in blood and urine from two sources: the use of alcohol-containing mouthwash and ingestion of nonalcoholic wine proven to contain EtG and EtS.

Materials and Methods

Study protocol

Twelve healthy volunteers (four men and eight women) with a median age of 22 years (range 19–30) and a median body mass index of 22.0 kg/m^2 (range 18.4–26.0) participated in a controlled drinking experiment. They were all social drinkers

with a median use of 12 standard drinks/month (range 4–20) and had abstained from alcohol during the week preceding the study, according to self-reports. Exclusion criteria were somatic or psychiatric illness and use of regular medication.

After an overnight fast, samples of oral fluid, blood, and urine were collected before start of the study. The participants were then divided into three groups. In the first group, four subjects rinsed their mouths with 15 mL of Listerine® mouthwash, containing 21.6% ethanol, for about 1 min and expectorated, followed by a 30-s break. This was repeated eight times, which resulted in a total amount of 120 mL over 5 min and exposure of a total dose of 20.7 g ethanol. The participants were instructed not to swallow the fluid. In the second group, another four subjects ingested one bottle (7.5 dL) of nonalcoholic wine (St. Regis Cabernet Sauvignon, Inglenook Vineyards, Madera, CA), which was determined by liquid chromatography–tandem mass spectrometry (LC–MS) to contain 3.0 mg/L EtG and 1.5 mg/L EtS. The intake occurred over a 1-h period. Two subjects only consumed 4.2 and 5.1 dL, respectively, due to nausea. This nonalcoholic wine also contained 0.2% ethanol, which was determined by headspace gas chromatography equipped with a flame-ionization detector (15). In the last group, the last four participants ingested a dose of ethanol approximately equivalent to that present in one bottle of nonalcoholic wine. Vodka containing 60% ethanol was used, and 3.75 mL was ingested in one gulp (dose 1.8 g ethanol). The vodka was analyzed by ultra-performance liquid chromatography (UPLC)–MS–MS and was confirmed to not contain EtG or EtS.

Samples of oral fluid, blood, and urine were collected at 1.5, 3.5, 5.5, and 7.5 h after intake. At the last time point, only samples of oral fluid and urine were collected. Oral fluid was collected using a Statsure Saliva Sampler (Saliva Diagnostic Systems, Brooklyn, NY). Urine was collected in Sterilin tubes without any additives, and for the blood samples, 5-mL Vacutainer tubes containing 20 mg fluoride and 143 I.U. heparin were used. All samples were stored at 4°C immediately after sampling, followed by storage at –20°C until analysis. Because of limitations in the analytical method, oral fluid samples were only analyzed for EtG (11), and blood and urine samples were analyzed for both EtG and EtS.

No food and drink apart from water was ingested until 1.5 h after start of intake. Then, meals consisting of bread, cheese, ham, and drinks were consumed at 1.5 and 5.5 h after intake. The participants were not allowed to consume any other food during the study period.

The subjects gave informed consent, and the study protocol was approved by the National Committee for Research Ethics in Norway and the Directorate for Health and Social Affairs.

Measurement of ethanol and creatinine

Alcohol was determined by an enzymatic method in oral fluid, blood, and urine (16). The creatinine concentration in urine was determined according to a previously published method (17).

Measurement of EtG in oral fluid

EtG was determined in oral fluid using a previously published method (11). Analysis of EtS in oral fluid was not possible with this method. The limit of detection (LOD) for EtG in oral fluid was 0.0022 mg/L, and the limit of quantification (LOQ) was 0.0044 mg/L. These values were for the oral fluid/buffer mixture.

Measurement of EtG and EtS in blood and urine

Chemicals. EtG and EtG-d_5 (internal standard) were supplied by MEDICHEM® (Steinenbronn, Germany). EtS and EtS-d_5 (internal standard) were supplied by Lipomed (Cambridge, MA). HPLC-grade methanol and acetonitrile for UV–HPLC were purchased from LAB-SCAN (Dublin, Ireland). Formic acid (98%) was purchased from VWR International (Fontenay sous Bois, France). Deionized water was obtained from a Milli-Q UF Plus water purification system (Millipore, Bedford, MA). Whole human blood was supplied from the blood bank at Ullevaal University Hospital (Oslo, Norway).

Preparation of standards. Stock standard solutions of EtG and EtS were prepared in methanol and working standard solutions were prepared in water from the stock solution. Spiked whole blood and urine samples were prepared from the working standard solutions at the concentration ranges: 0.1–20 mg/L in blood and 1.1–44 mg/L in urine for EtG, 0.03–6 mg/L in blood and 0.6–25 mg/L in urine for EtS.

Sample preparation. For blood, an aliquot of 200 µL whole blood was added to 50 µL internal standard solution (11 mg/L EtG-d_5 and 3 mg/L EtS-d_5) and 1 mL cold methanol. The samples were immediately agitated for 1 min and put in the deep-freezer for a minimum of 10 min. The samples were centrifuged at 4°C at 4500 rpm for 10 min. The methanol layer was transferred to a 5-mL glass tube and evaporated to dryness at 50°C under N_2. The residue was reconstituted with 60 µL acetonitrile/25 mM formic acid mixture (1:99, v/v), frozen, and centrifuged again at 4500 rpm for 10 min. The supernatant was transferred to autosampler vials.

For urine, an aliquot of 100 µL urine was added to 100 µL internal standard solution (same as previously) and 400 µL water. The samples were mixed for 10 min and centrifuged at 4°C at 4500 rpm for 10 min. Clear extract was transferred to the autosampler vials.

Instrumentation. The samples were analyzed by UPLC–MS–MS. LC was performed using an integrated system from Waters (Milford, MA) with an Acquity™ Ultra Performance LC. Chromatographic separation was performed at 65°C on an Acquity UPLC® HSS T3 column (2.1 × 100 mm, 1.8-µm particle size) using gradient elution with a mobile phase consisting of 25 mM formic acid (A) and methanol (B). The flow rate was 0.4 mL/min. A gradient was carried out starting from 1% B, increased to 20% over the next 2 min, then increased to 90% within 0.01 min and maintained for 2.99 min before returning to its initial conditions within 0.5 min. Total run time was 3.5 min. Injection volume was 2.5–3 µL.

MS detection was performed on a Waters Quattro Premier XE triple-quadrupole MS. Ionization was achieved using electrospray in the negative mode (ESI^-) and multiple reaction monitoring (MRM). The source block temperature was 120°C, and the capillary voltage was 1 kV. The desolvation gas (nitrogen) was heated to 400°C, and the flow was set to 1000 L/h.

The cone gas (nitrogen) was delivered at a flow rate of 50 L/h. In the collision cell, the collision gas (argon) flow was 0.35 mL/min, and the pressure was 9.5^{e-3} mbar. Data acquisition, peak integration, and calculation were interfaced to a computer workstation running MassLynx 4.1 SCN627 software (Waters). The following MRMs were used for detection: EtG 221.0 > 84.7, EtS 124.7 > 96.7, EtG-d_5 226.0 > 85.0, and EtS-d_5 129.7 > 97.7. Cone voltages were 35 V for both analytes and internal standards. The retentions time was 1.8 min for EtG and EtG-d_5 and 1.1 min for EtS and EtS-d_5.

Validation. Quantitative results were obtained by integrating the peak height of the specific MRM trace in reference to the integrated height of the internal standard. LOD and LOQ in blood were 0.03 mg/L and 0.06 mg/L for EtG and 0.007 mg/L and 0.02 mg/L for EtS, respectively. In urine, LOD and LOQ were 0.17 mg/L and 0.37 mg/L for EtG and 0.06 mg/L and 0.16 mg/L for EtS, respectively. The values were calculated as a mean of background noise + 3 standard deviations (SD) and +10 SD, respectively. Day-to-day variations were in the range of 8–14% (EtG: 0.12 and 13.5 mg/L, EtS: 0.04 and 4.2 mg/L) in blood and 11–12% (EtG: 1.3 and 22.4 mg/L, EtS: 0.8 and 12.7 mg/L) in urine. Intraday variations at the same concentration levels were between 3.6% and 8.7% in blood and 1.8% and 5.6% in urine. The EtG and EtS calibration curves were linear with correlation coefficients greater than 0.9996 for both analytes.

For EtG and EtS results in oral fluid, blood, and urine, results greater than LOD are reported as positive, and results less than LOD are reported as negative. Quantitative results are only reported if they were more than LOQ.

Statistics. All data were handled using the Kinetica (version 4.4) pharmacokinetic program (Thermo Fisher Scientific, Waltham, MA). Statistic parameters were calculated using SPSS (version 14.0, Chicago, IL).

Results

One subject in the nonalcoholic wine group (the one only ingesting 5.1 dL) showed EtG in urine before the start of drinking and was, therefore, excluded. Otherwise, all samples were negative for ethanol, EtG, and EtS before start of the study.

Results from blood and oral fluid

All samples of blood and oral fluid were negative for ethanol, EtG, and EtS in all three groups.

Results from urine

All samples were negative for ethanol in all three groups.

In the group ingesting nonalcoholic wine, all three subjects were negative for EtG in urine but positive for EtS (Table I).

Of the four subjects ingesting 3.75 mL 60% vodka in one gulp, two subjects showed positive samples of EtG and EtS in urine. The Cmax values in the first subject were 0.62 mg/L for EtG and 0.16 mg/L for EtS (normalized to 1000 mg/L creatinine), and this was reached after 3.5 h. The creatinine concentration was high in this sample (3028 mg/L). In the second subject, the Cmax values were 0.29 mg/L for EtG and 0.23 mg/L for EtS, and this was also reached after 3.5 h. The two other subjects ingesting this dose of ethanol were negative for EtG and EtS in urine.

All urine samples in the four subjects using mouthwash were negative for EtG and EtS.

Discussion

This study showed that accidental alcohol exposure did not lead to a false-positive EtG or EtS results in blood or oral fluid. It also showed that direct ingestion of EtG and EtS from nonalcoholic wine did not result in positive EtG but did show EtS concentrations up to 2.15 mg/L in urine.

False positives for EtG were neither found in blood nor oral fluid. This was as expected, considering the much lower concentrations of EtG in blood and even lower concentrations in oral fluid compared to urine (4,11). Therefore, the use of a higher cutoff level in oral fluid is not necessary, and the lack of such a source of error is an advantage when using oral fluid instead of urine to verify alcohol ingestion by the use of EtG.

This study also showed that the direct ingestion of EtG did not lead to positive results in oral fluid, blood, or urine, while direct ingestion of EtS did lead to positive results in urine. The amount directly ingested was approximately 2 mg EtG and 1 mg EtS, corresponding to the amount obtained after ingestion of 10–15 g of ethanol, according to studies which measured the total fraction of EtG and EtS excreted in urine (4). After such a dose of ethanol, positive levels of EtG in urine would be expected because concentrations between 0.4 and 3.7 mg/L were previously found after ingestion of 9 g ethanol (20). In another study, concentrations of EtG ranged between 0.6 and 8.4 mg/L after ingestion of 7 g of ethanol in a fasted state (21). The results from the present study therefore indicate that EtG ingested orally does not have 100% bioavailability. Regarding

Table I. Urine Samples Positive for EtS in the Three Subjects Ingesting Nonalcoholic Wine

Subject*	Time After Start of Drinking (h)	EtG	EtS (mg/L)†
1	1.5	negative	0.25
	3.5	negative	1.16
	5.5	negative	0.88
	7.5	negative	0.66
2	3.5	negative	1.30
	5.5	negative	2.15
	7.5	negative	1.12
3	3.5	negative	1.12
	5.5	negative	1.03
	7.5	negative	0.66

* Subjects 1 and 2 ingested 7.5 dL, and subject 3 ingested 4.2 dL.
† Concentrations normalized to 1000 mg/L creatinine.

EtS, 9 g of ethanol has led to concentrations of EtS between 2.0 and 11.1 mg/L (22), and the present work indicates enhanced bioavailability for EtS compared to EtG. The reason for this is unknown, but differing sizes and other properties of the molecule could be a possible explanation. These results are important in the practical use of EtG and EtS as relapse markers and show that EtS, which was previously assumed to be the most reliable of the two metabolites (23,24), could be falsely increased in urine after ingestion of nonalcoholic wine (25).

The ingestion of 3.75 mL vodka (1.8 g) was marginally larger than the amount present in one bottle of nonalcoholic wine and was performed to make up for the ethanol present in nonalcoholic wine. Considering our findings, the positive EtS results after the nonalcoholic wine ingestion are most likely caused by the directly ingested EtS, not conversion from the ethanol present as the EtS levels obtained were approximately ten times higher than the EtS levels obtained after drinking the actual amount of ethanol. On the other hand, we cannot exclude that a very small fraction of the EtS level was a result of conversion from alcohol.

Also, we showed that ingestion of a minimal dose of ethanol did yield positive EtG and EtS results in urine in two out of four subjects. Considering a previous publication where up to 0.35 mg/L EtG in urine was seen after ingestion of 1 g of ethanol (26), this was likely to happen. One could question why this dose of ethanol resulted in positive findings, while ingestion of the same amount of ethanol from the nonalcoholic wine did not. One explanation was that the ingestion of the nonalcoholic wine occurred over a 1-h period, which resulted in lower peak concentrations of ethanol compared to the same dose ingested in one gulp. Also, the actual urine samples had relatively high concentrations of creatinine, which could be another explanation for the positive results.

This study did not confirm the previous positive results of EtG in urine after intense use of mouthwash. Our LOD and LOQ in urine (0.17 and 0.37 mg/L, respectively) are similar to previously published levels (0.1–0.3 mg/L) after incidental alcohol exposure (12,13), but the exposure to the alcohol might have been less intense. Our laboratory has also set a cutoff level for EtG in urine (1.1 mg/L), as a result of the previous publications regarding use of mouthwash. The present study showed that this cutoff level was high enough to avoid such false-positive results for EtG in urine. But according to previous studies, it would not shorten the detection times for proper alcohol ingestion with a long time period (5,27).

The possibility of positive EtG results after incidental ethanol exposure, such as use of mouthwash, is an equivalent to the detection of morphine after ingestion of poppy seeds, which occurs in urine but not in blood. On the other hand, ingestion of poppy seeds has led to detection of morphine in oral fluid (28). This is not surprising considering the high pK_a value, excellent transfer of opiates into saliva, and subsequent higher concentrations in oral fluid (8). In contrary, EtG has a much lower pK_a value (3.21) (29), which leads to much lower concentrations in oral fluid. Positive results in oral fluid were therefore less likely.

In conclusion, this study showed that the possibility of false-positive results for EtG is unlikely in oral fluid and blood. EtG in oral fluid could therefore be used to detect alcohol ingestion in, for instance, workplace drug testing without fearing false-positive results after accidental alcohol exposure. Also, we showed that the bioavailability after direct oral ingestion is better for EtS than for EtG. False-positive urine result for EtS but not for EtG could therefore be seen after ingestion of nonalcoholic wine.

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