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ORIGINAL ARTICLE

Influence of Gilbert's syndrome on the formation of ethyl glucuronide

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Abstract A drinking experiment with participants suffering from Gilbert's syndrome was performed to study the possible influence of this glucuronidation disorder on the formation of ethyl glucuronide (EtG). Gilbert's syndrome is a rather common and, in most cases, asymptomatic congenital metabolic aberration with a prevalence of about 5 %. It is characterized by a reduction of the enzyme activity of the uridine diphosphate glucuronosyltransferase (UGT) isoform 1A1 up to 80 %. One of the glucuronidation products is EtG, which is formed in the organism following exposure to ethanol. EtG is used as a short-term marker for ethyl alcohol consumption to prove abstinence in various settings. After 2 days of abstinence from ethanol and giving a void urine sample, 30 study participants drank 0.1 L of sparkling wine (9 g ethanol). 3, 6, 12, and 24 h after drinking, urine samples were collected. 3 hours after drinking, an additional blood sample was taken, in which liver enzyme activities, ethanol, hematological parameters, and bilirubin were measured. EtG and ethyl sulfate (EtS), another short-term marker of ethanol consumption, were determined in the urine samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS); creatinine was measured photometrically. In all participants, EtG and EtS were detected in concentrations showing a wide range (EtG: 3 h sample 0.5–18.43 mg/L and 6 h sample 0.67–13.8 mg/L; EtS: 3 h sample 0.87–6.87 mg/L and 6 h sample 0.29–4.48 mg/L). No evidence of impaired EtG formation was found. Thus, EtG seems to be a suitable marker for ethanol consumption even in individuals with Gilbert's syndrome.

Keywords Ethyl glucuronide · Ethyl sulfate · Gilbert's syndrome · Uridine diphosphate glucuronosyltransferase

Introduction

Two decades ago, ethyl glucuronide (EtG) as a direct ethanol consumption marker and ethanol glucuronidation product was discussed for the first time [1]. Since then, its formation and excretion kinetics, sensitivity, and specificity as well as its fields of application have been the object of research [2–10]. Due to suitable characteristics and easy analysis by immunochemistry as well as liquid chromatography, EtG has crossed the threshold from science to routine use [11]. In combination with ethyl sulfate (EtS), it is used for monitoring individuals for abstinence from ethanol, e.g., in the withdrawal treatment of patients addicted to alcohol, medical/psychological examinations for regaining one's driving license, workplace drug testing programs or prior to liver transplantations [12–14].

For different issues, in large parts of the world numerous tests for EtG are performed. Since these tests may have forensic implications, they must be of high standards regarding sensitivity and specificity. False positive test results were

Dedicated to Prof. Dr. Stefan Pollak on the occasion of his 65th birthday

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obtained after unintentional or unknowing ingestion of ethanol in food, drinks, or sanitary products, like mouthwash, or after alcoholic fermentation in vivo with consecutive glucuronidation [8–10]. In contrast, bacterial degradation resulted in false negative test results [6, 7]. In drinking experiments, some individuals attracted attention by non-formation of ethyl glucuronide and therefore false negative assessments [5].

The reason for the absence of EtG formation has not been identified so far. A possible explanation could be a glucuronidation disorder: Gilbert's syndrome is a common congenital metabolic aberration [15]. Due to a polymorphism of the 5′ end of the gene promoter, the enzyme activity of the uridine diphosphate glucuronosyltransferase (UGT) isoform 1A1 is reduced by up to 80 % [16]. It is the most common glucuronidation disorder with a prevalence of about 7 % [17]. In most cases, the syndrome is asymptomatic; possible symptoms are mild intermittent jaundice, particularly of the sclerae, and fatigue [17]. Jaundice is ascribed to an increase in the level of unconjugated bilirubin, which occurs due to the reduced ability of glucuronidation. The disorder does not only affect the conjugation of bilirubin but also the glucuronidation of (therapeutic) drugs [16]. Irinotecan bears a higher risk of toxicity in affected persons; other substrates of UGTs are paracetamol and oxazepam [15]. The diagnosis is commonly made by exclusion, when neither hemolysis nor a hepatocellular disease can explain the high bilirubin concentrations. It can be confirmed, e.g., by molecular genetic tests and liver biopsy [15–17]. No specific therapy is necessary, and prognosis is good [17]. Persons suffering from Gilbert's syndrome fully participate in daily life and, as the case may be, it can happen that they are submitted to abstinence monitoring programs.

The aim of our study was to investigate a possible influence of Gilbert's syndrome on the formation of EtG. Impaired glucuronidation of ethanol may lead to positive discrimination of this group of persons. It seems possible that EtG is not detectable in persons with Gilbert's syndrome due to a severe disorder of glucuronidation (not only of ethanol). The detection of the phase II metabolites EtG and EtS in this particular group of persons was the primary aim of our study. For this purpose, a drinking experiment with participants suffering from Gilbert's syndrome was performed. The setting of this drinking study was the same as in a previous experiment with healthy volunteers [18].

Materials and methods

Experimental setup

The study was approved by the Ethics Committee of Freiburg University (project nr. 303/12). All study participants gave their written informed consent. The subjects were recruited from doctors' surgeries and via newspaper ads. The diagnosis was confirmed by a medical certificate and/or laboratory results.

The drinking experiment was performed as follows: After at least 2 days of abstinence from alcoholic beverages and the collection of a void urine sample, 30 participants with Gilbert's syndrome aged 18 to 71 years (18 males, 12 females) were asked to drink 0.1 L of sparkling wine within 5 min. Body weight and height of all test persons were recorded, and every participant completed a questionnaire to provide further information about their age, medical history, medication, and general drinking behavior.

Apart from the void sample, urine samples were collected 3, 6, 12, and 24 h after ethanol consumption. A longer period was not considered necessary, because the study was focusing directly on the formation or non-formation of EtG. Blood samples were taken 3 h after drinking.

EtG, EtS, and creatinine were determined in the urine samples. Blood samples were used to measure the blood ethanol concentration and to perform blood tests (blood count, conjugated as well as total bilirubin and liver enzymes). The blood tests were performed in the Department of Clinical Chemistry using routine methods.

Determination of the blood ethanol concentration

Blood ethanol concentrations were measured using HS-GC-FID (headspace gas chromatography-flame ionisation detection) with t-butanol as internal standard. Ethanol determination was performed using linear calibration with aqueous calibrators containing 0.1, 0.2, 0.5, 1, 2, 3, 4, and 5 g/L of ethanol. The lower limit of quantitation (LLOQ) was the lowest calibrator's concentration (0.1 g/L or 0.08 g/kg for blood).

Determination of EtG and EtS

Chemicals and instrumentation

EtG was obtained from Medichem (Steinenbronn, Germany). Ethyl sulfate, pentadeuterated ethyl sulfate (D_5-EtS), and pentadeuterated EtG (D_5-EtG) were purchased from Lipomed (Weil am Rhein, Germany). Methanol was delivered by Sigma-Aldrich (Taufkirchen, Germany). Formic acid was obtained from AppliChem (Darmstadt, Germany) and 2-propanol from Carl Roth GmbH & Co.KG (Karlsruhe, Germany). All solvents were of analytical or HPLC grade.

Analysis was performed with a liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) system consisting of a QTrap 2000 triple quadrupole mass spectrometer (AB SCIEX, Darmstadt, Germany) equipped with a TurboIonSpray™ interface and an Agilent 1100 series HPLC-system (Agilent, Waldbronn, Germany). Chromatographic separation was

performed on a polar-endcapped phenylpropyl reversed phase column (Synergi Polar-RP 250×2 mm, 4 μm) with matching guard column (4 mm×2 mm, packing material identical; Phenomenex, Aschaffenburg, Germany).

Sample preparation

0.1 μg D_5-EtG and D_5-EtS were added as internal standards to 100 μL of urine. 250 μL of methanol were added for protein precipitation, and the samples were centrifuged for 10 min at 13,000*g*. 270 μL of the supernatant were separated and evaporated in a vacuum centrifuge. The dried extracts were reconstituted with 250 μL of 0.1 % aqueous formic acid, and 20 μL were injected into the LC-MS/MS system.

EtG and EtS quantification

HPLC separation was performed at 40 °C with isocratic elution (0.1 % formic acid/methanol) at a flow rate of 0.2 mL/min. To increase the volatility of the eluent, 2-propanol was added post-column at a flow rate of 0.2 mL/min via a T-union. EtG and EtS were analyzed simultaneously using a validated MRM method [6, 19]. The method was calibrated from 0.08 to 10 mg/L for EtG and 0.1 to 2 mg/L for EtS. The lowest calibration level corresponds to the lowest limit of quantitation (LLOQ) in urine, which was determined according to forensic guidelines [20]. The limit of detection was 0.05 mg/L for EtG and EtS. The following MS/MS transitions with precursor ion/product ion were used: EtG: *m/z* 221/75 as quantifier, 221/113 and 221/85 as qualifiers, and *m/z* 226/75 for D_5-EtG; EtS: *m/z* 125/97 as quantifier, *m/z* 125/80 as qualifier, and *m/z* 130/98 for the deuterated internal standard. Criteria for compound identification were based on forensic standards [5, 19]. Analyst® QuantitationWizard (V.1.5; AB SCIEX, Darmstadt, Germany) software was used for quantitation. To balance urine dilution, the obtained EtG and EtS concentrations were normalized to 100 mg/dL creatinine (EtG_{100}, EtS_{100}).

Determination of creatinine

Creatinine was determined using the Jaffé reaction (DRI® Creatinine-Detect® Test, Microgenics, Passau, Germany) with a Konelab 30i Analyzer (Thermo Scientific, Dreieich, Germany).

Results

Thirty subjects aged 18 to 71 years (mean 38.9 years) participated in this study. Twelve of them were female and 18 were male. Body height ranged from 163 to 194 cm (mean 171 cm), and body weight was between 41 and 86 kg (mean 67.9 kg). The respective body mass indices were 15.4 to 27.3 (mean 21.7).

According to the questionnaire, none of the participants suffered from a severe medical condition; in their medical history, the participants mentioned allergies, hypothyreosis, hypertension, and depressive episodes. Medications for these conditions as well as oral contraception were stated with regard to drugs taken. Six study participants reported that they rarely drink alcoholic beverages. Ten subjects stated to drink mainly wine (between one glass and two bottles per week), while 13 participants prefer beer (between 2 and 10 bottles/week). Only one subject (#5) stated in the questionnaire that he regularly drinks larger amounts of alcoholic beverages (at least two drinks/day, more on weekends).

In none of the participants, severe abnormalities of the blood count were found. There was no evidence of hemolysis. Aspartate transaminase was within the normal range in all subjects. Participant #5 (male, 29 years, BMI 23.7) showed a slight increase of alanine transaminase (54 U/L, normal range 10–50 U/L). In five participants (#5, 7, 22, 28, 30), increased concentrations of γ-glutamyl transpeptidase were observed. Subjects #5 and 28 stated a daily consumption of one or two alcoholic beverages as a possible explanation for the increased levels. In the other participants, neither the information provided on ethanol consumption nor any known liver diseases gave an explanation for the higher concentration.

Twenty-one of the 30 participants showed an increased concentration of total bilirubin. The unconjugated bilirubin level was within the normal range in nine subjects. These participants submitted written medical certificates confirming the diagnosis. For unconjugated bilirubin, values up to 3.3 mg/dL were found.

Apart from one participant (#29), all void samples were negative for EtG and EtS. This "void" sample was the only urine specimen with higher EtS_{100} than EtG_{100} concentrations. In 23 subjects, the highest concentrations of EtG_{100} were observed in the sample taken 3 h after drinking. Six persons showed EtG_{100} peak concentrations 6 h after drinking. Only for one participant (#11) was the highest EtG_{100} concentration measured in the 12 h post consumption sample after a decrease of EtG and EtS levels from the 3 h sample to the 6 h sample. Only three subjects showed the peak concentration of EtS_{100} in the sample taken 6 h after drinking; in all the other participants, the maximum concentrations were observed in the first urine sample collected after drinking (3 h).

The concentrations of EtG_{100} and EtS_{100} showed a wide inter-individual range (Fig. 1a, b): The minimum and maximum concentrations for EtG_{100} and EtS_{100} are indicated in Table 1. Apart from subject #11 with a second increase of EtG_{100} and EtS_{100} levels in the sample taken 12 h after drinking, EtG could still be detected in 6 and EtS in 4 participants after 24 h (Fig. 2).

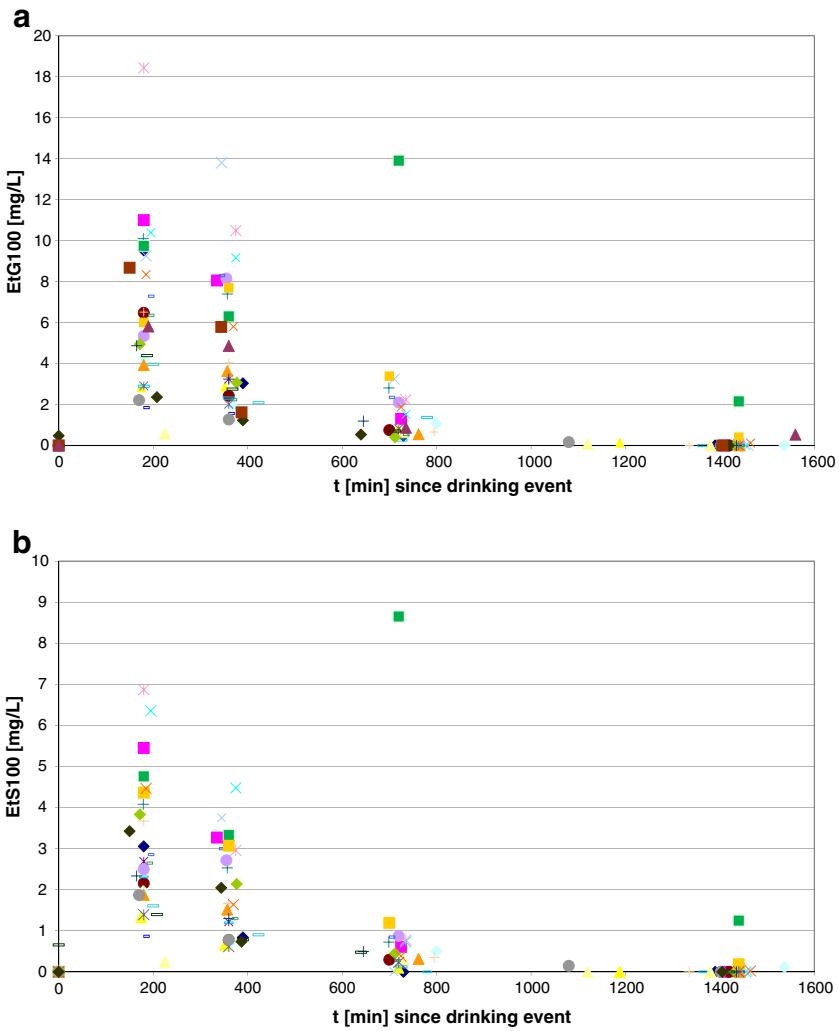

Fig. 1 **a** Range of EtG_{100} concentrations [mg/L]. **b** Range of EtS_{100} concentrations [mg/L]

Discussion

In participant #29, the urine sample obtained prior to drinking ethanol contained EtG and EtS (EtG_{100}: 0.48 mg/L; EtS_{100}: 0.66 mg/L). The question was whether the period of 2 days was too short or abstinence was not maintained. In the self-disclosure questionnaire, this participant stated an average ethanol consumption of one bottle of beer (0.5 L) per day. EtG and EtS formed from this amount should be excreted within the period of abstinence fixed for this study. Within the study, there was no evidence of an impaired excretion of EtG and EtS.

In one participant (#11), marker concentrations rose again from the sample taken after 6 h to the 12 h sample. A second ethanol ingestion of about the same dose has to be suspected. Assuming that within the first few hours after the study drinking period, no further alcohol was ingested, the person reached rather high marker concentrations (EtG_{100} 9.74 mg/L after 3 h, 6.3 mg/L after 6 h, 13.9 mg/L after 12 h, and 2.15 mg/L after 24 h; EtS_{100} 4.76 mg/L after 3 h, 3.33 mg/L after 6 h, 8.65 mg/L after 12 h, and 1.25 mg/L after 24 h). There was no peculiarity or medication indicated by this subject that could serve as a source of ethanol.

From the very beginning of research on EtG kinetics, a wide range of metabolization rates was noticed. The marker

Table 1 Concentration ranges for EtG_{100} and EtS_{100}

	EtG_{100} [mg/L]		EtS_{100} [mg/L]	
Sampling time after drinking [h]	Minimum	Maximum	Minimum	Maximum
3	0.5	18.43	0.87	6.87
6	0.67	13.8	0.29	4.48
12	0.08	3.39 (13.9)	n.d.	1.19 (8.65)
24	n.d.	0.53 (2.15)	n.d.	0.19 (1.25)

Concentrations in parentheses refer to proband #11 with a second increase of EtG and EtS concentrations

n.d. not detectable

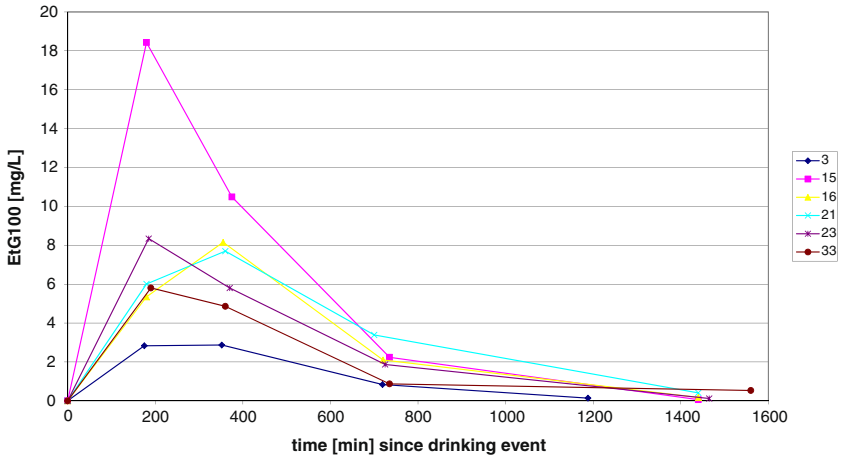

Fig. 2 EtG_{100} concentrations [mg/L] in participants with marker detectability 24 h after drinking

concentrations in this study also vary markedly. For this particular experiment, the same amount of alcohol was chosen for every participant; the dose of ethanol was not related to the body weight. Maximum and minimum concentrations of EtG_{100} and EtS_{100} for the 3 h urine sample differed by factors 36 and 7.9, respectively.

Maralikova et al. [18] performed a drinking experiment with the same amount of ethanol (9 g) and choice of beverage. Eleven participants were included and three to four urine samples were collected over a period of 3 days. In that study, the EtG_{100} concentrations ranged between 0.2 and 3.1 mg/L. In our experiment, the EtG_{100} concentrations varied more distinctly (measured minimum and maximum concentrations 0.5 and 18.4 mg/L, respectively). The maximum values were observed between 2.7 and 10 h after ethanol intake. Conditions regarding time cannot account for this difference, since the relevant time periods are covered in both studies. The most plausible explanation is the higher number of participants in this recent study accompanied by larger inter-individual variation.

Interestingly, the period of detectability also varied considerably and did not depend on the level of the maximum concentrations. In six subjects, EtG could still be detected after 24 h without indication of any further ethanol consumption. While the maximum concentrations in four of these participants were above the median (6.01 mg/L), the measured maximum EtG_{100} levels in two participants were below the median (#3 and 14). In these two persons, the long period of detectability cannot be due to high concentrations. Creatinine levels that could indicate dehydration were not particularly high either. These findings may be ascribed to inter-individual variations in kinetics.

As regards the initial question, whether Gilbert's syndrome influences the formation of the ethanol glucuronidation product EtG, no dysfunction can be deduced from the results of this study. All participants in this experiment formed and excreted EtG as well as EtS. The maximum EtG_{100} concentrations even exceeded those formerly published [18].

The enzyme uridine diphosphate glucuronosyltransferase is subclassified into several isoforms. In Gilbert's disease, isoform 1A1 is affected. In experiments with recombinant UGTs, Foti and Fisher observed the highest formation rate of EtG for this particular isoform (1A1) [21]. Multiple isoforms possess the ability for ethanol glucuronidation [21, 22]. Schwab and Skopp obtained maximum EtG formation rates with the recombinant human UGT 1A9 and 2B7 [22]. The plurality of EtG catalyzing isoforms seems to be the reason for the detectability of this ethanol consumption marker in persons with a particular glucuronidation disorder. Concerns were raised that EtG may not be a valid marker in individuals with Gilbert's disease. The results of this study do not give an indication that EtG could not be suitable, though.

Several limitations of this study have to be taken into consideration. This study was performed with the primary aim to detect EtG and EtS in persons with diagnosed Gilbert's syndrome. A direct control group was not studied; the experiment was performed according to a previous drinking study, and the results were compared [18]. For inclusion into the study, a written medical report of the diagnosis of Gilbert's syndrome by a physician was required, but no molecular genetic tests to confirm the diagnosis were carried out.

Conclusions

EtG was detected in all participants and seems to be a suitable marker for monitoring abstinence even in persons suffering from the glucuronidation disorder known as Gilbert's disease. Due to the glucuronidation capability of various isoforms, a mutation of the 1A1 isoform of uridine diphosphate glucuronosyltransferase—as claimed for Gilbert's syndrome—obviously does not affect the total

formation of ethyl glucuronide. The formation of EtS is not influenced by Gilbert's syndrome either.

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Conflicts of interest There are no conflicts of interest to declare.

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