Int J Legal Med (2008) 122:123–128
DOI 10.1007/s00414-007-0180-8

ORIGINAL ARTICLE

Kinetics in serum and urinary excretion of ethyl sulfate and ethyl glucuronide after medium dose ethanol intake

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Received: 7 December 2006 / Accepted: 10 May 2007 / Published online: 9 June 2007
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Abstract The direct ethanol metabolites, ethyl glucuronide (EtG) and ethyl sulfate (EtS), are of increasing importance for clinical and forensic applications, but there are only few studies on the kinetics of EtG in serum and none on EtS. In this study, 13 volunteers (social drinkers) drank ethanol in the form of white wine to reach a blood alcohol concentration of 0.51±0.17 g/kg, and blood and urine samples were analyzed for EtG and EtS simultaneously by chromatography-tandem mass spectrometry (LC-MS/MS). Mean peak serum EtG and EtS concentrations were 2.9±1.3 and 2.8±1.6 μmol/l, respectively, and were reached between 4.0±0.9 h after the start of drinking (3.0±0.5 h for EtS). The mean time differences between reaching maximum blood ethanol levels and serum metabolite levels were 2.3±0.9 h for EtG and 1.2±0.5 h for EtS. In the last blood samples collected (10–11 h after the start of drinking), 11 (of 13) volunteers were still positive for EtG in serum, whereas only 2 were positive for EtS. In the serum of one female person, no EtS was detectable at any time; however, it was excreted in the urine in (low) concentrations. Ethanol was detectable in the serum for up to 8.6 h after the start of drinking, whereas EtG and EtS were detectable up to more than 5.8 h (EtG) and 4.0 h (EtS), respectively. Mean peak urinary concentrations were 401±232 μmol/l for EtG and 266±153 μmol/l for EtS, and mean peak levels were reached 6.2±0.9 h (EtG) and 5.3±1.2 h (EtS) after the start of drinking. Maximum concentrations of EtG and EtS in serum showed a wide interindividual variation and could not be correlated to the maximum blood ethanol concentrations. Correlations ($p<0.001$, Kendall's Tau *b*) were found when comparing pairs of parameters, but mostly involved areas under the curve (AUC) of metabolites or of ethanol; one correlation linked the peak concentrations of EtG and EtS in urine.

Keywords Ethyl glucuronide · Ethyl sulfate · Ethanol markers · Kinetics · LC-MS/MS

Introduction

Biological markers are becoming more and more important to prove ethanol consumption due to the growing impact of alcoholism on modern societies. Most of the ethanol consumed is oxidized to acetaldehyde and acetic acid, but a small part undergoes nonoxidative transformation. The nonoxidative, direct metabolites, ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidyl ethanol (PEth), and fatty acid ethyl esters (FAEE) can be used as markers for alcohol consumption besides classical state markers such as carbohydrate deficient transferrin (CDT), gamma glutamyl transferase (GGT), or mean corpuscular volume (MCV) [18]. Furthermore, EtG and EtS close the gap in the detection window between short-term markers (e.g., ethanol) and long-term markers like CDT, GGT, and MCV. EtG has a far longer detectability time than ethanol itself [15–17], of up to 80 h in urine. In literature, there has been a discussion

Electronic supplementary material The online version of this article (doi:10.1007/s00414-007-0180-8) contains supplementary material, which is available to authorized users.

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about stability of ethanol and EtG postmortem and about generation of ethanol due to putrefaction without generation of EtG, which can be used for interpretation of postmortem ethanol concentrations [11]. EtS seems to have a similar urinary excretion profile as EtG, but further studies with larger numbers of volunteers are needed to find valid correlations [3, 6, 10]. Interindividual variations of the formation of EtG and EtS can be explained by considerable polymorphisms in the genes coding for enzymes responsible for EtG and EtS synthesis, namely, uridine diphosphate (UDP)-glucuronosyltransferases, mainly UGT1A1 and UGT2B7, [2, 5] and sulfotransferases [12, 13]. This study was performed to obtain more knowledge about the formation and excretion kinetics of both EtG and EtS in blood and urine.

Materials and methods

Participants and specimen collection

The study was approved by the Ethics Commission of the University of Freiburg (201/02-05). Informed consent was obtained before the beginning of the experiments.

For this study, 13 healthy volunteer social drinkers (age 19–42 years; six females, seven males; BMI 18–29.5 kg/m^2; see Table 1) who abstained from alcohol for at least 1 week gave blood samples 2 days before the experiment to obtain normal hemograms [i.e., mean corpuscular volume MCV, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), gamma-glutamyl transferase (GGT), which were all within normal physiological limits]. On the first day of the experiment, blank urine and blood samples were taken before the volunteers were asked to consume an amount of alcohol, which would lead to a blood ethanol concentration of 0.5–0.8 g/kg, calculated by the Widmark equation corresponding to sex, weight, and height [1]. The start of drinking was directly after a standardized breakfast (roll with jam or honey, caffeine-free tea and coffee) and drinking took about 30 min.

From 1–5 h after the start of drinking, venous blood samples were taken at intervals of 30 min, from 5–10 h at intervals of 60 min. Urine samples were obtained approximately every 2 h until 10 h after the start of drinking, followed by three to four samples evenly distributed over the next day (day 2) and the morning urine of day 3. Approximately 3.5 and 9.5 h after the start of drinking, the volunteers were given standardized meals (after 3.5 h pizza and mineral water, after 9.5 h bread with sausage and cheese and mineral water). After the last blood sample, the volunteers were sent home with instructions to abstain from ethanol contained in drinks, food, or medications until the end of urine sampling.

Reagents and instrumentation

High performance liquid chromatography (HPLC)-grade acetonitrile and formic acid (analytical grade) were obtained from Merck (Darmstadt, Germany). EtG and pentadeuterated EtG (D_5-EtG) were purchased from Medichem (Stuttgart, Germany). Sodium ethyl sulfate was obtained from ABCR (Karlsruhe, Germany). Deuterated ethyl sulfate (D_5-EtS) was prepared by an in-house procedure [3].

Mass spectrometric analyses were performed with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system consisting of an API 365 triple quadrupole tandem mass spectrometer with a Turbo IonSpray interface (Applied Biosystems/Sciex, Darmstadt, Germany) and a HPLC system (three pumps LC-10AD; system controller SCL-10A Shimadzu, Duisburg, Germany). HPLC separation was achieved at 40°C with a polar-endcapped phenylpropyl reversed phase column (Synergy Polar-RP 250×2 mm, 4 μm) with a guard column (4 mm×2 mm, same packing material; Phenomenex, Aschaffenburg, Germany). For isocratic elution, 0.1% formic acid was used with a flow rate of 0.2 ml/min. Acetonitrile was added post-column at a flow rate of 0.2 ml via a T union to enhance the volatility of the eluent. Both EtG and EtS were analyzed in one run by a validated MS/MS method with electrospray ionization (ESI) [14]. The method was calibrated from 0.45 to 225 μmol/l for EtG and from 0.79 to 397 μmol/l for EtS. The limits of quantitation (LOQ) in urine (0.45 μmol/l for EtG and 0.79 μmol/l for EtS) had been determined during method validation (using VALISTAT 1.0 software, Arvecon GmbH, Walldorf, Germany) according to forensic guidelines. For serum, the same LOQ was used (0.45 μmol/l for EtG and 0.79 μmol/l for EtS), as the signal-to-noise ratio for EtG in serum was 12 at 0.45 μmol/l, and for EtS 10 at 0.79 μmol/l. Intraday precision with serum samples was 11.8% (at 3.28 μmol/l, $n=6$) and 8.1% (at 1.90 μmol/l, $n=7$) for EtG, respectively, and 11.0% (at 2.26 μmol/l, $n=7$) and 13.3% (at 0.88 μmol/l, $n=7$) for EtS, respectively.

For EtG, the MS/MS transition with *m/z* 221/75 (precursor ion/product ion) was used as quantifier, *m/z* 221/203, 221/113, and 221/85 were used as qualifiers and *m/z* 226/75 represented the deuterated standard. The transitions for EtS were *m/z* 125/97 (quantifier), 125/80 (qualifier), and 130/98 for the deuterated standard.

Ethanol in urine and serum was determined in duplicate both by headspace-gas chromatography with flame ionization detector (GC-FID) with tertiary butanol as internal standard and enzymatically using an alcohol dehydrogenase method (Cobas Mira S, Roche, Mannheim, Germany with DRI® Ethyl Alcohol Assay 0037, Microgenics, Passau, Germany). The serum concentrations have been converted to blood alcohol concentrations according to forensic

guidelines by use of the conversion factor 0.809. Urinary creatinine was determined by a Roche Hitachi 902 (Roche Diagnostics, Mannheim, Germany) analyzer using the Jaffé reaction (DRI® Creatinine-Detect® Test, Microgenics, Passau, Germany). Data were processed with Microsoft Excel 2002. Statistic parameters (correlation coefficient, significance level) were calculated using SPSS 13.0.1.

Sample preparation

Urine or serum samples (100 µl) were spiked with 30 µl of a ready-made mixture of D_5-EtG and D_5-EtS (containing 0.1 µg D_5-EtG in 30 µl standard mixture and a constant amount of D_5-EtS synthesized by an in-house procedure [3]), proteins were precipitated from the mixture with 250 µl methanol for urine and 250 µl acetonitrile for serum samples, respectively. After centrifugation, 270 µl of the supernatant was evaporated in a vacuum centrifuge and reconstituted with 140 µl of 0.1% aqueous formic acid (280 µl for urine samples with a creatinine concentration higher than 135 mg/dl), and 10 µl aliquots were injected into the LC-MS/MS system.

Results

The blank blood and urine samples obtained before the start of drinking were all negative for ethanol, EtG, and EtS. The volunteers consumed between 0.50 and 0.78 g ethanol/kg body mass (see Table 1). The resulting mean peak EtG and mean peak EtS concentrations in serum were 1.2–4.9 µmol/l (mean 2.9±1.3 µmol/l SD, relative SD 43%, median 2.8 µmol/l) and 1.0–6.4 µmol/l (2.8±1.6 µmol/l, 59%, 2.3 µmol/l), respectively (see Table 2, Fig. 1). Peak serum EtG concentrations were reached between 2.3 and 5.0 h (4.0±0.9 h, 21%, 4.25 h) after the start of drinking, peak EtS concentrations after 2.1–3.9 h (3.0±0.5 h, 17%, 2.9 h).

Peak blood alcohol concentrations (BAC) were reached between 1.3 and 2.1 h (1.8±0.2 h, 13%, 1.9 h). Differences of times for reaching mean peak BAC and mean peak Se ETS concentrations were between 0.5 and 2.0 h (1.2±0.5 h, 42%, 1.2 h). Differences of times for reaching peak BAC and peak Se ETG concentrations were between 0.5 and 3.5 h (2.3±0.9 h, 38%, 2.1 h). Differences of times for reaching peak EtS and peak EtG concentrations in serum were between 0 and 2.4 h (1.2±0.9 h, 71%, 1.3 h) (see Table 2).

In seven volunteers, higher molar peak EtS concentrations were found, but in contrast in six persons, the molar peak EtG concentration was higher than the peak EtS concentration. Eleven of the volunteers still had EtG in the last blood sample more than 10 h after the start of drinking, whereas only two volunteers had an EtS positive last

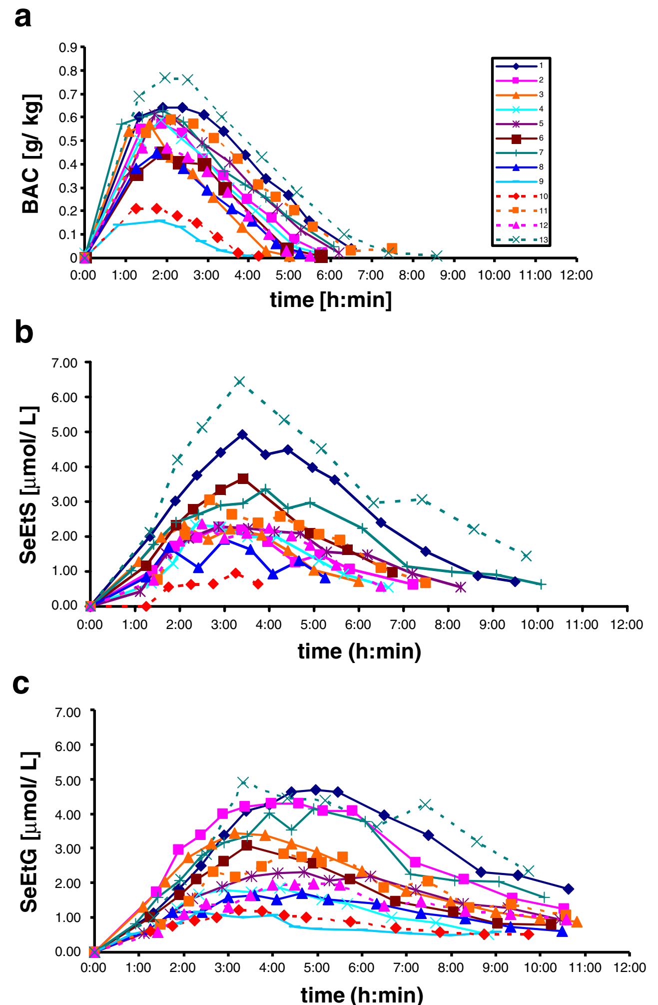

Fig. 1 Concentrations of ethanol in blood (**a**), EtS in serum (**b**), and EtG in serum (**c**) (*from top to bottom*) after consumption of 0.50–0.78 g EtOH/kg body weight

sample. Remarkably, in the serum samples of one volunteer (no. 9), no EtS could be detected, although EtG and ethanol were detectable. In the remaining ten volunteers, the last EtS-positive serum sample was drawn between 3.8 and 9.5 h after the start of drinking. The molar ratio of peak EtS/EtG ranged from 0 to 1.31 (0.90±0.35, relative SD 39%, median 1.01). Comparison of peak serum EtG and EtS concentrations from two volunteers indicated, for volunteer 3, that the peak EtS concentration was 60% of the EtG concentration, whereas volunteer 13 showed a 1.3 times higher EtS concentration than EtG (see Fig. 2).

Mean peak urinary concentrations of 104–805 µmol/l (401±232 µmol/l, 58%, 409 µmol/l) for EtG and 46–533 µmol/l (266±153 µmol/l, 57%, 283 µmol/l) for EtS (see Table 2, Fig. 3) were found, which peaked between 5.0 and 7.5 h (6.2±0.9 h, 14%, 6.2 h) (EtG) and 3.1 and 7.4 h (5.3±1.2 h, 22%, 5.5 h) (EtS). In 12 volunteers, the molar peak urinary concentration of EtG was higher than that of

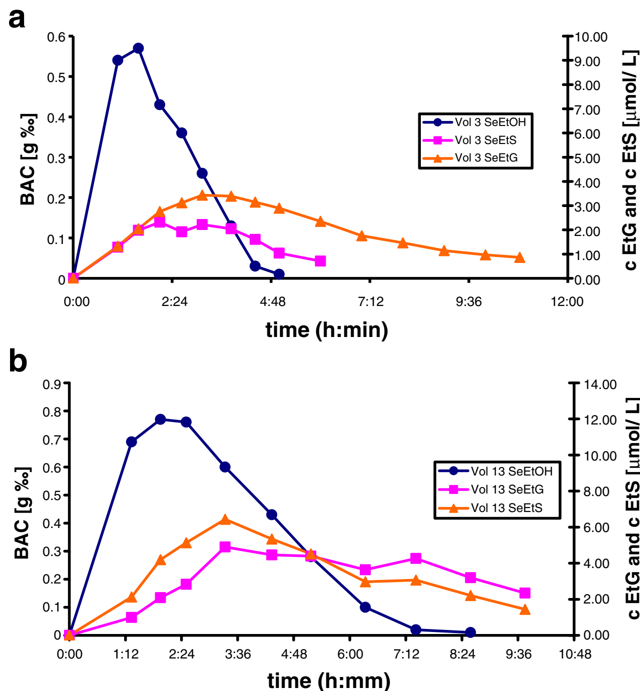

Fig. 2 **a**, **b** Blood ethanol (BAC), serum EtS and EtG in two volunteers. **a** volunteer 3 (0.52 g EtOH/kg body weight), **b** volunteer 13 (0.78 g EtOH/kg body weight)

EtS. Volunteer 4 showed more urinary EtS than EtG. Four of the subjects still had EtG in the last urine sample (longer than 44 h), and the other nine persons had the last EtG-positive urine sample between 26.6 and 36.1 h after the start of drinking. EtS showed a slightly different profile, as one person had an EtS-positive urine in the last sample after 70 h, but this person showed a significant increase in EtS levels after 42 h, although there was no increase in EtG. Two persons still excreted EtS in urine after more than 47 h, and from the remaining ten urine samples, EtS had disappeared between 22.8 and 35.8 h after the start of drinking. The ratio of molar concentrations of EtS and EtG ranged from 0.44 to 1.24 (0.70±0.25, 35%, 0.69). Also in urine, large interindividual differences in peak EtG and EtS concentrations were observed. The peak EtS concentration of volunteer 3 was 28% of the peak EtG concentration, whereas it was 88% of the peak EtG concentration in volunteer 8.

The following parameters have been checked for correlation in pairs: peak concentrations of EtG, EtS, and ethanol in serum, peak concentrations of EtG, EtS, and ethanol in urine, areas under the curve (AUC) of EtG, EtS, and ethanol in serum, consumed amount of alcohol, consumed amount of alcohol per kg body weight, age, height, body mass, and body mass index (BMI).

Correlations were found for ethanol, EtG, and EtS, when comparing serum peak concentrations and AUCs. In addition, seven highly significant correlations ($p \leq 0.001$) were found (see Table 3), using the model of Kendall's Tau b correlation. This statistical model was applied, as the variables were not normally distributed, had a moderate linear relationship, and because the Kendall Tau b model is advantageous when outliers occur.

For serum, the AUC of ethanol correlated with the peak concentration of EtG (correlation coefficient 0.753; significance level $p<0.001$) and also with the AUC of EtG (0.735; $p<0.001$) and the AUC of EtS (0.857; $p<0.001$). Correlations were also found between AUC of EtS and the peak concentration of EtG (0.805; $p<0.001$) and also with the peak concentration of ethanol (0.737; $p=0.001$), and with the AUC of EtG (0.761; $p<0.001$). For urine, there was a correlation between the peak concentration of EtG and the peak concentration of EtS (0.684; $p=0.001$).

In Table 2, the mean peak blood and urine concentrations of ethanol, serum and urine EtG and EtS are compared. As expected, the SDs for ethanol both in urine and blood were smaller than those for EtG and EtS. One volunteer even showed no detectable amount of EtS in serum, whereas ethanol and EtG were found in blood and EtS and EtG in urine as well. Peak ETG concentration in this volunteer's serum was 40% of the average value of all

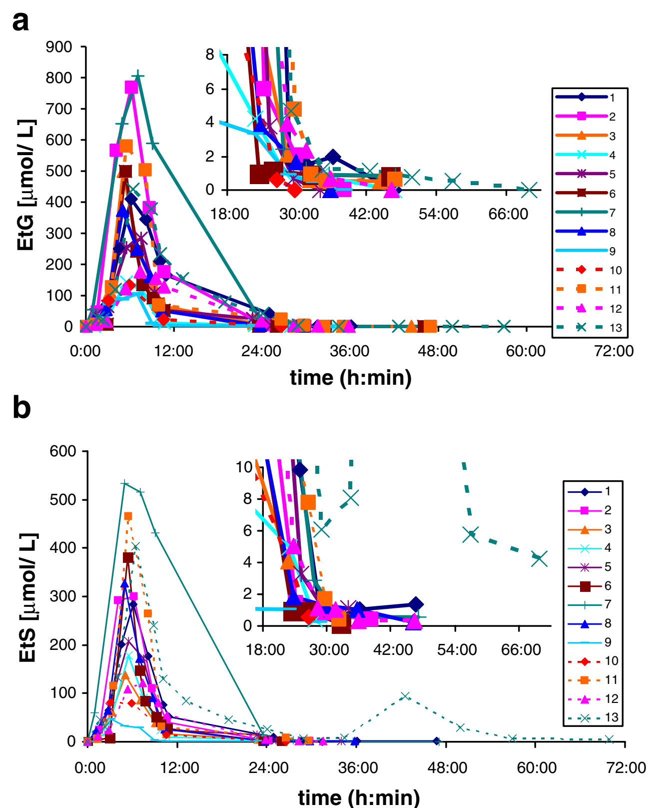

Fig. 3 Concentrations of EtG (**a**) and EtS (**b**) in urine after consumption of 0.50–0.78 g EtOH/kg body weight

volunteers, peak EtS concentration in urine 26%, and peak EtG concentration in urine 18% of the average value, respectively.

Discussion

EtS and EtG were detectable in urine and serum for a longer period of time than ethanol itself. In serum, EtS was detectable for about twice as long as ethanol, and EtG, for even longer. In urine, EtG was detectable for up to 10 times longer than ethanol, EtS, 3–8 times longer. There were large variations in the time differences for reaching maximum concentrations of blood alcohol, serum EtG, and serum EtS. Some correlations could be found, but most of them involved at least one AUC, which is not very useful in spot sample applications. The only fully non-AUC correlation occurred between the maximum concentrations of EtG and EtS in urine.

In former studies, it has been shown that glucuronyltransferases and sulfotransferases show polymorphism [5, 13], and furthermore, induction of the glucuronyltransferase UGT1A1 by alcohol has been reported [8]. By inclusion of enzymatic formation, distribution, and elimination, a kinetic model for EtG was recently postulated by Droenner et al. [4], but none has been calculated for EtS, yet. However, our study clearly shows that there are huge interindividual differences particularly in sulfoconjugation. In addition to polymorphisms of conjugating enzymes, differences in their activity, or expression, the sulfation might be influenced by other factors, such as nutrition as proposed in the twin study of Nash et al. [9]. Therefore, and due to the relatively low number of individuals tested, we could not describe a new kinetic model for EtS applicable for all volunteers. Furthermore, we detected an unexpected increase in urinary EtS after 42 h in volunteer 13 who had shown “normal” formation and urinary excretion of both EtG and EtS in parallel in several preceding drinking studies. This person was compliant concerning abstinence from ethanol and an incidental uptake of alcohol leading to 94 μmol/l EtG in urine would also have raised the EtG levels, which was not observed, and therefore, can be excluded. The reason for this increase has to be found elsewhere, e.g., uptake of EtS with food or nonalcoholic beverages. To prove this, further studies on this aspect have to be performed. To collect information for further evaluation of both markers, we included both markers in our routine analysis method, which is used for monitoring compliance of patients in ethanol withdrawal therapy and for forensic purposes.

Testing for EtG and EtS in parallel—which is not more time-consuming than EtG alone—is recommended for forensic cases, as excretion in urine is qualitatively very similar, although interindividual variances do occur. Furthermore, stability issues, due to bacterial contamination caused by e.g., urinary tract infections have been raised, which resulted in a degradation of EtG but not EtS [7].

Acknowledgements The authors wish to thank the “Bund gegen Alkohol und Drogen im Straßenverkehr e.V.” for the funding of the study.

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