

ASSESSMENT AND DETECTION

Detection Times for Urinary Ethyl Glucuronide and Ethyl Sulfate in Heavy Drinkers during Alcohol Detoxification

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Abstract — **Aims:** Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are conjugated ethanol metabolites formed in low amounts after alcohol consumption. Compared with ethanol, EtG and EtS are excreted in urine for a prolonged time, making them useful as sensitive alcohol biomarkers. This study determined the detection times for EtG and EtS in alcoholic patients undergoing alcohol detoxification. **Methods:** Alcohol-dependent patients ($n = 32$) with an initial alcohol concentration ≥ 1 g/L based on breath testing were followed during detoxification. Urine samples for determination of EtG, EtS, ethanol and creatinine were collected on admission to the hospital and thereafter once daily for several days. EtG and EtS measurements were performed by liquid chromatography-mass spectrometry (LC-MS) and EtG also using an immunochemical assay (DRI-EtG EIA, ThermoFisher/Microgenics). **Results:** The detection time for urinary EtG was weakly correlated ($r = 0.434$, $P = 0.013$) with the initial alcohol concentration (range 1.0–3.4 g/L). For EtG, the individual time range until return to below the applied cut-off limit (<0.5 mg/L) was ~ 40 – 130 h (median 78) with a similar time course observed for EtS. After correction for urine dilution, the time until an EtG/creatinine ratio <0.5 mg/g was ~ 40 – 90 h (median 65). The detection times after an estimated zero ethanol concentration were ~ 30 – 110 h (median 66) for EtG and ~ 30 – 70 h (median 56) for EtG/creatinine. The EtG results by LC-MS and the immunoassay were in good agreement. **Conclusions:** During alcohol detoxification, EtG and EtS remained detectable in urine for several days. The detection times showed wide inter-individual variations, also after adjusting values for urine dilution and to the estimated times for a completed ethanol elimination.

INTRODUCTION

Following alcohol intake, an absolute majority ($>95\%$) of the ethanol becomes oxidized by alcohol dehydrogenase to acetaldehyde and further to acetic acid by aldehyde dehydrogenase. Because of rapid ethanol metabolism and excretion from the body, the time frame for a positive saliva, breath or blood ethanol test is typically limited to <12 h and some hours longer in urine, owing to retention of urine in the bladder (Helander *et al.*, 1996). To aid in the detection of excessive and harmful alcohol consumption, much research effort has focused on developing sensitive alcohol biomarkers with a longer detection window than what is possible by ethanol testing (Helander, 2003).

A small amount ($<0.1\%$) of the ethanol ingested becomes conjugated with glucuronic acid and sulfate to form ethyl glucuronide (EtG) (Schmitt *et al.*, 1995; Dahl *et al.*, 2002) and ethyl sulfate (EtS) (Helander and Beck, 2004), respectively. These phase-II reactions are catalysed by UDP-glucuronosyltransferase (Foti and Fisher, 2005) and sulfotransferase (Schneider and Glatt, 2004). After drinking alcohol, EtG and EtS are excreted for considerably longer time than ethanol (Schmitt *et al.*, 1995; Dahl *et al.*, 2002; Sarkola *et al.*, 2003; Borucki *et al.*, 2005; Helander and Beck, 2005), and urine testing for these minor ethanol metabolites has hence gained popularity as a sensitive method to spot recent alcohol intake (Helander, 2003; Politi *et al.*, 2007). The presence of EtG and EtS provides a strong indication of recent drinking, even if ethanol is no longer detectable (Helander and Beck, 2005). EtG has been recommended for use in clinical and forensic investigations of alcohol intake (Wurst *et al.*, 2003; Skipper

et al., 2004; Erim *et al.*, 2007; Hoiseth *et al.*, 2007a; Kugelberg and Jones, 2007) and utilized to document abstinence in treatment programs, for random alcohol testing in workplaces and schools, and as proof of drinking by courts.

It should be noted that EtG, but not EtS, can be produced post-sampling, if specimens are infected with *E. coli* (the primary pathogen in urinary tract infection) and contain ethanol (e.g. formed by fermentation in samples from diabetics) (Helander *et al.*, 2007). Considering the potential serious consequences of a false-positive clinical result, caution is therefore always advised when interpreting the results from EtG testing. The United States Substance Abuse and Mental Health Services Administration has issued an advisory warning against using a positive EtG as primary or sole evidence of drinking for disciplinary and legal action (Center for Substance Abuse Treatment, 2006). Furthermore, EtG (Helander and Dahl, 2005; Hoiseth *et al.*, 2007b), but not EtS (Helander and Dahl, 2005), is sensitive to bacterial hydrolysis if infected samples are stored improperly, implying a risk also for obtaining false-negative results.

Detailed knowledge about the detection windows for EtG and EtS after drinking is a fundamental requirement, when these metabolites are used for clinical and medico-legal purposes. Studies conducted on healthy volunteers have established the detection times following intake of low-to-medium doses of ethanol under standardized conditions. Typically, EtG and EtS are detectable in urine for ≤ 24 h after intake of ≤ 0.25 g/kg ethanol, and for ≤ 48 h after intake of ≤ 0.50 g/kg ethanol (Dahl *et al.*, 2002; Helander and Beck, 2005; Wojcik and Hawthorne, 2007; Hoiseth *et al.*, 2007a, 2008; Halter *et al.*, 2008). Also consumption of very small ethanol doses (≤ 10 g) is detectable

for many hours afterwards (Stephanson *et al.*, 2002; Helander and Beck, 2005; Wurst *et al.*, 2006) and even unintentional intake from the use of ethanol-based mouthwash (Costantino *et al.*, 2006) and hand sanitizers (Rohrig *et al.*, 2006) could yield a positive urinary EtG and EtS, if applying a very low analytical cut-off limit. In blood, the corresponding detection times are considerably shorter (e.g. ≤ 14 h at 0.5 g/kg) (Schmitt *et al.*, 1997; Hoiseth *et al.*, 2007a; Halter *et al.*, 2008).

Much less information is available on the detection windows for EtG and EtS after heavy intoxication (Wurst *et al.*, 2002; Borucki *et al.*, 2005). An initial study suggested detection times for urinary EtG up to ~ 75 h (Alt *et al.*, 1997) and, more recently, detection times for EtG ranging from < 24 h to > 90 h were demonstrated in alcohol-dependent patients during recovery from heavy drinking (Beck *et al.*, 2007). The present work was conducted to establish the detection windows for EtG and EtS in urine in alcoholic patients during alcohol detoxification and to examine factors that could possibly be of influence.

MATERIALS AND METHODS

Patients and sampling

Randomly selected alcohol-dependent patients (meeting DSM IV criteria since 0.5–34 years, mean 19.1) being hospitalized at Universitätsklinikum Mainz, Klinik für Psychiatrie und Psychotherapie, Germany, for alcohol detoxification participated in this study. Their alcohol consumption in the last week ranged between 300 and 4380 g (mean 1650, median 1600) according to self-report. During the first 3 days of inpatient treatment, they were under strong supervision and could not leave the ward. From Day 4 onwards, they were allowed to have visitors and also to leave the ward for some time. In case of alcohol withdrawal symptoms, patients were treated with clomethiazole (Lange-Asschenfeldt *et al.*, 2003).

Patients showing an ethanol concentration ≥ 1 g/L (based on breath measurement) on admission and a markedly lower or negative value at the second testing carried out in the next morning on average 18 h later, confirming that they were in the elimination phase and had not ingested ethanol in-between (Norberg *et al.*, 2003), were included. The first urine sample was collected on admission to the hospital, a second urine sample in the next morning and thereafter once every ~ 24 h (first morning voids whenever possible) over several days (5–8 samples/patient, mean 7.3). Urine specimens were collected in plastic urine monovettes without preservatives (Sarstedt AG, Germany) and stored at -20°C until analysis. On each urine sampling, breath alcohol measurement was done in parallel. Breath tests and clinical observations were used to control for abstinence during the study period.

The study was approved by the ethics committee at the University of Mainz.

Measurement of EtG, EtS, ethanol and creatinine

Measurement of EtG and EtS in urine was done by a sensitive and specific liquid chromatographic-mass spectrometric (LC-MS) method (Stephanson *et al.*, 2002; Helander and Beck, 2004, 2005). The analysis was performed in the negative-ion mode, using selected ion monitoring of the deprotonated ions at m/z 221 and m/z 226 for EtG and the penta-deuterated internal standard (EtG-D5; Medichem Diagnostics, Germany), and at

m/z 125 and m/z 130 for EtS (TCI, Japan) and EtS-D5 (internal standard) (Helander and Beck, 2005), respectively. The detection and quantification limits of this method are ~ 0.05 and 0.1 mg/L, respectively, but for clinical purposes, a cut-off limit of 0.5 mg/L EtG ($2.2 \mu\text{mol/L}$) is routinely applied (Böttcher *et al.*, 2008), while EtS is used as a confirmatory test (limit of quantification 0.1 mg/L). All EtG-positive results by LC-MS were confirmed by LC-MS/MS (Perkin–Elmer 200 LC system and Sciex API 2000 MS) by the presence of the correct relative abundance of the major product ions of EtG (m/z 75, 85 and 113). No influence by ion suppression was noted at the retention times of the analytes (Stephanson *et al.*, 2002; Helander and Beck, 2005).

Determination of urinary EtG was also performed using a commercial enzyme immunochemical method for EtG (DRI-EtG EIA, ThermoFisher/Microgenics, Germany), as detailed elsewhere (Böttcher *et al.*, 2008).

Breath ethanol measurement was performed using a Dräger Alcotest model 7410 (Dräger Safety AG, Germany) and the results were expressed as the corresponding concentration in blood (quantification limit 0.05 g/L). The urinary ethanol concentration was determined by the alcohol dehydrogenase method on an Olympus AU640 (Olympus, Germany) with reagents from ThermoFisher/Microgenics (quantification limit 0.1 g/L).

Creatinine measurement was done with the Jaffé method on an Olympus AU640 with reagent from ThermoFisher/Microgenics.

Statistics

Statistical calculations were performed using the *F*-test for comparison of variance and Pearson's correlation coefficient (MedCalc software version 9.3.9.0).

RESULTS

Altogether 32 alcohol-dependent patients (31 men and 1 woman) aged 31–72 years (mean 46, median 46) with a body

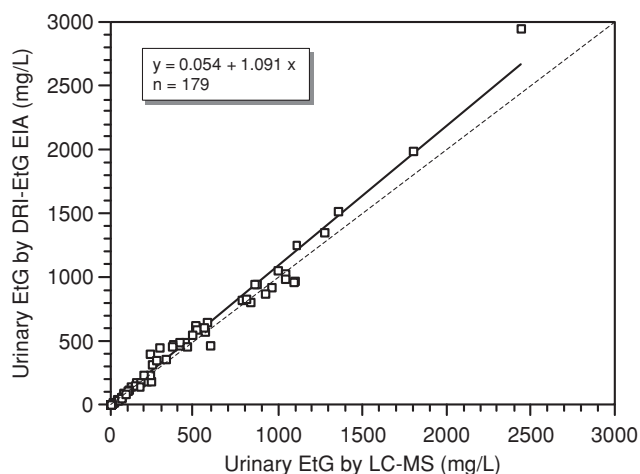


Fig. 1. Agreement of urinary ethyl glucuronide (EtG) levels determined by LC-MS and the EtG-DRI (Microgenics/ThermoFisher) immunoassay. Only data for EtG above the quantification limit of the LC-MS method (~ 0.1 mg/L) are shown.

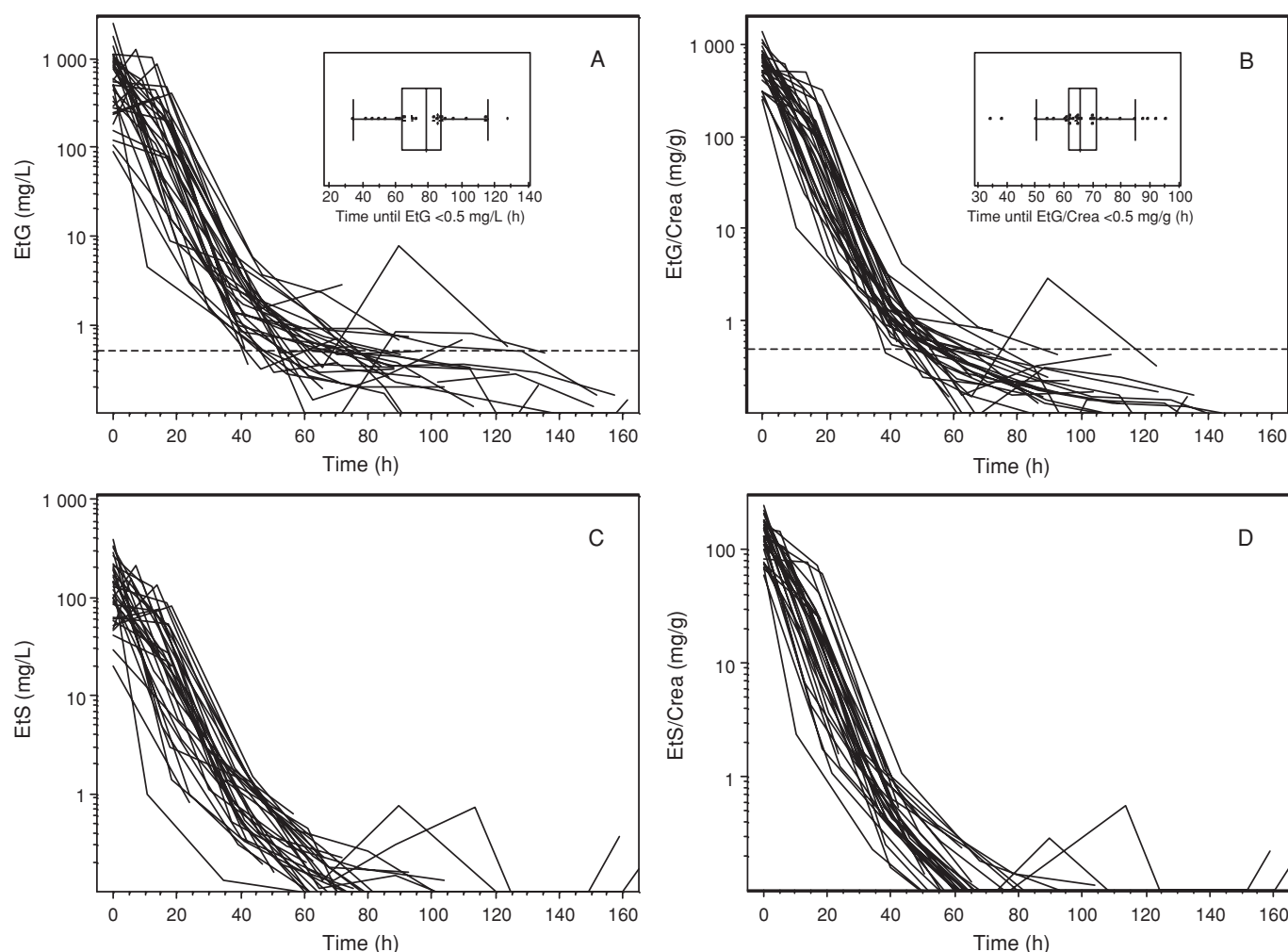


Fig. 2. Urinary excretion time profiles for EtG and EtS concentrations (A and C) and the corresponding values after normalizing the results to urinary creatinine (B and D) in 32 alcohol-dependent patients during detoxification (5–8 samples/patient, mean 7.3). Inset: Box-and-whisker plots for the times until urinary EtG and EtG/creatinine had returned to below the cut-off limits (<0.5 mg/L and <0.5 mg/g, respectively).

weight of 41.5–124 kg (mean 77.0, median 75.8) fulfilled the inclusion criteria of this study, by showing an ethanol concentration of at least 1 g/L on admission to the hospital and a markedly lower or negative second test result carried out on average 17.6 h (median 16.8, range 5.5–41.5) later. Breath alcohol measurements demonstrated initial ethanol concentrations ranging from 1.0 g/L to 3.4 g/L with a mean value of 2.0 g/L (median 1.9).

Measurement of urinary EtG and EtS was carried out by LC-MS with all positive results confirmed by LC-MS/MS. For comparison, EtG was also quantified using the DRI-EtG enzyme immunoassay. An overall good agreement of the EtG results obtained with the DRI-EtG and LC-MS/MS was seen over the entire concentration range (0–2440 mg/L) (Fig. 1). In the calculations, however, only the LC-MS/MS data were employed.

Individual time course graphs for urinary EtG and EtS are shown in Fig. 2. For all 32 patients, EtG and EtS remained positive for considerably longer time than urinary ethanol (ethanol data not shown). All urine samples collected on admission to the hospital were positive for ethanol (range 1.0–4.4 g/L, mean

3.0, median 3.1) but in only four (12.5%) patients, ethanol was also detectable in the second urine sample collected the next morning. For EtG, the time from admission (i.e. first testing) until return to below the applied clinical cut-off limit (<0.5 mg/L) ranged from ~40 h to ~130 h (Fig. 2A) with a mean of 77 h (median 78, 25th–75th percentile 64–88). A similar time course was seen for urinary EtS with detection times of ~55–110 h when using a quantification limit of 0.1 mg/L (Fig. 2C). The EtG and EtS values showed an overall good correlation with an EtG/EtS mean molar ratio of 2.5 (Fig. 3).

In at least two of the patients, increased EtG and EtS values were seen after the return to low or negative values, indicating some kind of ethanol exposure (either drinking on purpose or due to unintentional ingestion from ethanol-containing products) (Fig. 2). However, no positive breath tests were recorded during the study period.

Because the EtG and EtS concentrations are known to be influenced by urine dilution (Dahl *et al.*, 2002; Bergström *et al.*, 2003; Helander and Beck, 2005), the effect of normalizing values to urinary creatinine was evaluated. This practice resulted in a lower inter-individual variability for both metabolites

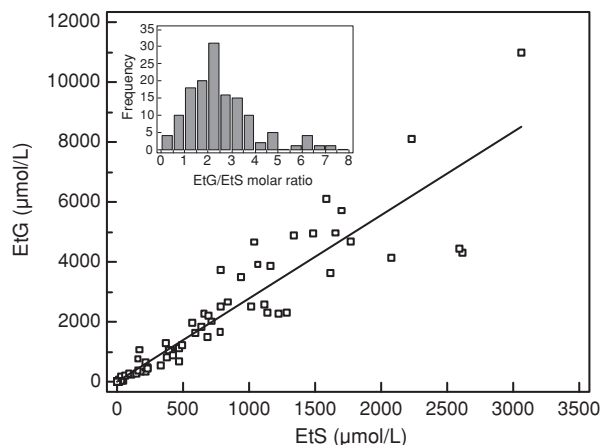


Fig. 3. Correlation between urinary EtG and EtS concentrations determined by LC-MS. Regression equation: $y = 5.974 + 2.786 x$, $n = 297$ (all values included), $r = 0.951$, $P < 0.0001$. Inset: the corresponding EtG/EtS molar ratio (mean 2.5, median 2.25, range 0.21–7.22).

(Fig. 2B and D). For EtG/creatinine (Fig. 2B), the time until return to below 0.5 mg/g ranged from ~40 h to ~90 h with a mean of 67 h (median 65, 25th–75th percentile 62–72).

A weak positive correlation was seen between the initial breath ethanol concentrations and the urinary detection times for EtG ($r = 0.434$, $P < 0.013$) but did not reach statistical significance for EtS ($r = 0.189$). To compensate for the variable initial alcohol concentrations and infrequent urine sampling times, the approximate time for a zero ethanol concentration was estimated for each patient, based on their ethanol concentration on admission and applying an average ethanol elimination rate of 0.18 g/L/h (Jones *et al.*, 1997). When expressed in this way, the excretion curves for EtG and EtS showed even better inter-individual agreement (Fig. 4A and C) (time range until EtG <0.5 mg/L ~30–110 h, mean 66, median 66, 25th–75th percentile 54–80; $P = 0.013$ compared with the original standard deviation). After also correcting for variations in urine dilution by calculation of ratios to urinary creatinine (Fig. 4B and D), the time range until EtG/creatinine was <0.5 mg/g was ~30–70 h (mean 56, median 56, 25th–75th percentile

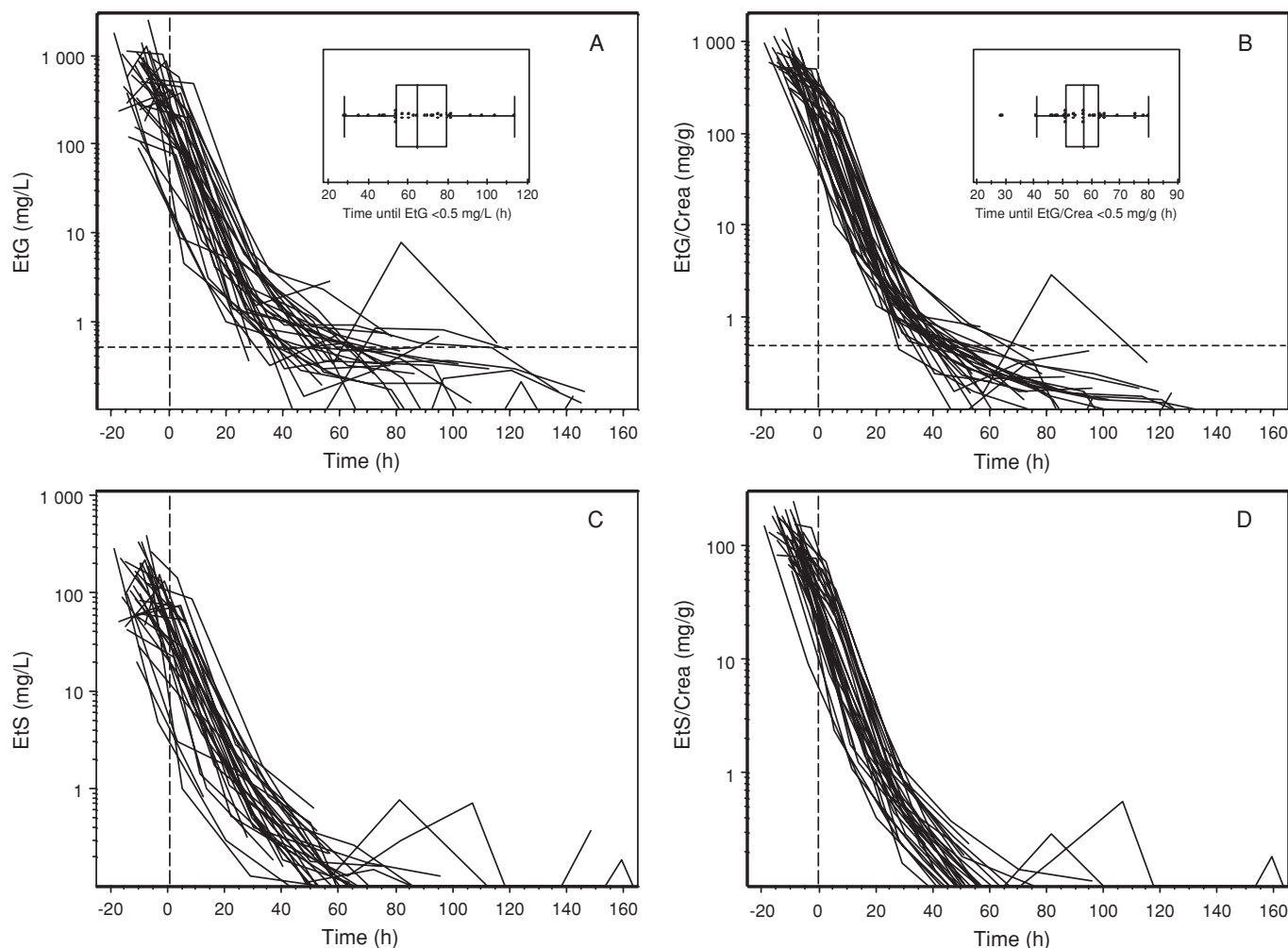


Fig. 4. Urinary excretion time profiles for EtG and EtS concentrations in relation to the estimated time for a zero ethanol concentration (A and C), and the corresponding values after normalizing the results to urinary creatinine (B and D) in 32 alcohol-dependent patients during detoxification (5–8 samples/patient, mean 7.3). Inset: Box-and-whisker plots for the times after the estimated zero ethanol concentration until urinary EtG and EtG/creatinine had returned to below the cut-off limits (<0.5 mg/L and <0.5 mg/g, respectively).

Table 1. Urinary detection times for EtG and EtS after different doses of alcohol

Population	N	Alcohol dose (g/kg)	Detection window (h) ^a		Cut-off limit (mg/L)		Reference
			EtG	EtS	EtG	EtS	
Healthy subjects	4	0.1	≤6	–	0.1	–	(Stephanson <i>et al.</i> , 2002)
	2	0.1	13–22	22–26	0.15	0.11	(Wurst <i>et al.</i> , 2006)
	9	0.15	–	≤12	0.1	–	(Helander and Beck, 2005)
	5	0.25	<24	–	0.1	–	(Wojcik and Hawthorne, 2007)
	6	0.2–0.3	3–25	5–30	0.15	0.11	(Wurst <i>et al.</i> , 2006)
	6	0.5	22–32	–	0.1	–	(Dahl <i>et al.</i> , 2002)
	10	0.5	25–35	–	0.2	–	(Hoiseth <i>et al.</i> , 2007a)
	9	0.5	–	≤24	0.1	–	(Helander and Beck, 2005)
	1	0.5	≤29	≤29	0.1	0.1	(Helander and Beck, 2004)
	10	0.5	25–48	25–39	0.1	0.1	(Hoiseth <i>et al.</i> , 2008)
	7	0.5	≤48	–	0.1	–	(Wojcik and Hawthorne, 2007)
	1	0.6	≤36	≤36	0.15	0.11	(Wurst <i>et al.</i> , 2006)
	13	0.50–0.78	27–44	23–47	0.1	0.1	(Halter <i>et al.</i> , 2008)
	7	0.75–0.85	≤48	–	0.1	–	(Wojcik and Hawthorne, 2007)
	17	>1	39–102	–	0.1	–	(Borucki <i>et al.</i> , 2005)
	12	Alcohol intoxication	≤75	–	0.1	–	(Alt <i>et al.</i> , 1997)
	32	Alcohol intoxication	40–130	55–110	0.5	0.1	Present study

^aIn studies on healthy subjects, the detection times after the start of drinking are usually reported, while in studies of alcoholic patients, the times after admission to the hospital are usually given.

51–63; $P = 0.004$ compared with the uncorrected standard deviation).

DISCUSSION

In recent years, EtG has attained increasing interest as a biomarker of acute alcohol intake with several potential clinical and medico-legal applications (Helander, 2003). With the advent of a commercial immunochemical reagent for rapid and cost-effective assay of urinary EtG (Böttcher *et al.*, 2008), this interest is even likely to increase. The first validation of the immunochemical DRI-EtG enzyme assay demonstrated good agreement with the LC-MS values (Böttcher *et al.*, 2008), and the present study confirmed these results and further supported the clinical usefulness of the immunoassay. Accordingly, it is now possible to perform EtG analysis according to the concept of screening and confirmation, as is a common practice in urine drug testing. Confirmation analysis of EtG is best performed by LC-MS/MS (Weinmann *et al.*, 2004). However, due to the risk for EtG degradation and/or artifactual formation in infected samples, EtS, which is indicated to be stable under such conditions (Helander and Dahl, 2005; Helander *et al.*, 2007), is recommended as a complementary validation parameter (Helander and Beck, 2005).

A number of studies have reported data on the detection times for EtG following the start of alcohol intake or, which is most important in the clinical situation, after the standard test for recent drinking (ethanol in breath or blood) is negative. These studies have mostly been conducted with healthy volunteers drinking standardized (e.g. adjusted for body weight) low-to-medium ethanol doses, but only occasionally examining clinical patients during detoxification after, presumably, much higher doses. In controlled studies with healthy volunteers, the excretion profiles for EtG and EtS in urine and blood are now well documented (Schmitt *et al.*, 1997; Dahl *et al.*, 2002; Wurst *et al.*, 2002, 2005; Borucki *et al.*, 2005; Helander and Beck, 2005) and have established a dose–response relationship for

peak values and, as summarized in Table 1, detection times. For example, ethanol doses between ~0.25 and 0.50 g/kg typically result in detection times for urinary EtG of ~24–48 h after the start of ethanol ingestion, or some hours shorter if instead expressed as the time after ethanol is no longer detectable in blood (Dahl *et al.*, 2002). In alcoholic patients followed during detoxification, detection times up to ~80 h were initially reported (Wurst *et al.*, 1999) and even longer times were found in a more recent study (Beck *et al.*, 2007). Still, a study of 23 alcoholic patients during detoxification suggested a typical detection time of only ~48 h after the ethanol had been eliminated (Wurst *et al.*, 2002). The results of the present study demonstrated highly variable inter-individual detection times for EtG after admission to the hospital, ranging from ~40 h up to ~130 h. The corresponding detection times for urinary EtS were in the same range (~55–110 h). It should be pointed out that the infrequent urine sampling (once daily) is likely to have added to this wide detection window.

EtG and EtS are excreted in the urine in a process influenced by water-induced diuresis (Dahl *et al.*, 2002; Goll *et al.*, 2002; Helander and Beck, 2005), making it possible to include correction to the urine creatinine concentration for some applications (Bergström *et al.*, 2003). Although the present results demonstrated that normalization of values for urine dilution, and for variable ethanol doses (i.e. estimated time for zero ethanol concentration), resulted in significantly less scattered individual elimination curves (Figs 2 and 4), a marked inter-individual variation in the concentration–time profiles still remained for both metabolites. In the clinical situation, where the times for ethanol intake and any urine voiding between drinking and sampling are usually not known, it will hence be impossible to link a single EtG and EtS result to a specific ethanol dose taken at a specific time.

No common cut-off or reporting limit has yet been agreed upon for urinary EtG and EtS when used as alcohol biomarkers. In this study, the routine in-house threshold limit at 0.5 mg/L EtG (roughly corresponding to 0.5 mg/g creatinine) was employed, because it allows unintentional exposure for ethanol

to remain undetected (Böttcher *et al.*, 2008), while EtS is routinely used as a confirmatory test with a quantification limit of 0.1 mg/L. Most LC-MS/(MS) methods using modern instruments would allow for a lower EtG cut-off limit to be applied, resulting in a somewhat longer detection window but at the same time an increased risk for obtaining false-positive clinical results due to unintentional ethanol exposure. A possibility not yet fully explored in routine practice is to normalize the measured levels to the creatinine concentration to compensate for intentional and unintentional urine dilution. Today, a lower dilution threshold for urine creatinine (usually 20 mg/dL) is often applied in urine drug testing (Fraser and Zamecnik, 2003), but it should be pointed out that this cut-off still allows for a significant intentional urine dilution to take place.

In conclusion, the present study carried out on alcohol-dependent patients during alcohol detoxification not only indicated that EtG and EtS may remain detectable in urine for several days after heavy drinking but also demonstrated large inter-individual variations, even after values were normalized for urine dilution and the variable initial ethanol concentrations (i.e. alcohol doses). Measurement of EtG is routinely applied in clinical and medico-legal drug testing. Considering the long detection time even after intake of low-to-moderate ethanol doses, it is very important to inform the customers that a positive result must not be interpreted as always resulting from heavy drinking and may not qualify for use in pre-employment and random drug testing. The feature of using urinary EtG and EtS testing for detection of heavy drinking several days back should preferentially be of value for detection of slip or relapse drinking in a treatment situation where complete abstinence from alcohol is required or agreed upon.

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