

Urinary Tract Infection: A Risk Factor for False-Negative Urinary Ethyl Glucuronide but Not Ethyl Sulfate in the Detection of Recent Alcohol Consumption, Anders Helander* and Helen Dahl (Department of Clinical Neuroscience, Karolinska Institutet and University Hospital, Stockholm, Sweden; * address correspondence to this author at: Alcohol Laboratory, L7:03, Karolinska University Hospital Solna, SE-171 76 Stockholm, Sweden; fax 46-8-51771532, e-mail anders.helander@cns.ki.se)

After consumption of alcoholic beverages, the bulk of the ethanol dose (95%–98%) is eliminated in a 2-stage oxidation process mainly in the liver, first to acetaldehyde by alcohol dehydrogenase and then further to acetic acid by aldehyde dehydrogenase. The remainder is excreted unchanged in urine, sweat, and expired air (1). In addition, a very small fraction (<0.1%) of the ingested ethanol undergoes phase II conjugation reactions to produce ethyl glucuronide (EtG) and ethyl sulfate (EtS) (2, 3), catalyzed by uridine diphosphate-glucuronosyltransferase or sulfotransferase, respectively. EtG and EtS are eventually excreted in the urine. As both of these nonoxidative direct ethanol metabolites show much longer elimination times than ethanol itself (3), the interest in EtG and EtS has focused largely on their use as sensitive and specific biomarkers of recent alcohol intake with clinical and forensic applications (4, 5). A positive finding of EtG and/or EtS provides a strong indication that the person was recently drinking alcohol, even when the ethanol concentration has returned to 0 or is no longer measurable.

Glucuronide and sulfate conjugates of endogenous and exogenous origin are cleaved by β -glucuronidase and sulfatase, enzymes that are widely distributed among animals and plants. β -Glucuronidase is also present with high activity in most strains of *Escherichia coli* (6). Because this characteristic is rather unique for *E. coli* compared with other bacterial species, β -glucuronidase assays with chromogenic and fluorogenic substrates have been devel-

oped for the rapid and specific identification of *E. coli* in clinical microbiological diagnostics and for testing contamination of food and water (7, 8). Sulfatase activity has been detected in many different bacteria (9), but not in *E. coli* (10, 11), or only in very low amounts (12).

E. coli is the most common bacterium isolated in clinical laboratories and is also the predominant pathogen (~80%) in urinary tract infections (UTIs) (13). This study, therefore, evaluated whether the presence of *E. coli* or other common pathogens in urine specimens, resulting from UTIs or possible contamination during sampling and handling, could give false-negative EtG and EtS results in the detection of recent alcohol consumption because of hydrolysis by bacterial β -glucuronidase and sulfatase.

Fresh clinical urine specimens (n = 46; stored refrigerated) containing confirmed bacterial growth at a density of 10^3 to $>10^5$ colony-forming units (CFU)/mL and with >80% of samples containing $>10^5$ CFU/mL, as identified by culture on standard solid media, were obtained from the microbiology laboratory at the Karolinska University Hospital. Specimens were collected consecutively from the routine pool of infected urine samples and were also selected to include different pathogens. The samples were supplemented with 1 mg/L each of EtG (Medichem Diagnostics) and EtS (TCI); they were then split into 3 tubes (without preservatives), which were placed at -20 , 4 , and 22 °C. Urine without the addition of EtG and EtS served as controls. At the start and after 1, 2, and 5 days of storage at 4 and 22 °C, samples were placed at -20 °C until taken for analysis of EtG and EtS by a sensitive and specific direct electrospray liquid chromatographic–mass spectrometric (LC-MS) method (3). LC-MS analysis was performed in the negative ion mode, with selected ion monitoring of the pseudomolecular ions at m/z 125 for EtS (M_r 126.1) and m/z 130 for EtS-D₅ (pentadeuterated internal standard, prepared by reaction of ethanol-D₆ with chlorosulfonic acid; Sigma-Aldrich) and at m/z 221 and m/z 226 for EtG (M_r 222.1) and EtG-D₅ (internal standard;

Table 1. Uropathogens tested for causing falsely low or false-negative EtG or EtS results as a result of hydrolysis by bacterial β -glucuronidase and sulfatase.

Uropathogen identified	Urine specimens tested, n	Specimens causing hydrolysis of EtG, ^a n (%)	Specimens causing hydrolysis of EtS, ^a n
<i>E. coli</i>	25	17 (68) ^b	0
<i>Enterococcus</i> sp.	5	0 (0)	0
Group B streptococci	3	0 (0)	0
<i>K. pneumoniae</i>	3	1 (33) ^c	0
<i>Proteus mirabilis</i>	2	0 (0)	0
<i>Pseudomonas aeruginosa</i>	2	0 (0)	0
<i>Staphylococcus saprophyticus</i>	2	0 (0)	0
<i>Citrobacter freundii</i>	1	0 (0)	0
<i>E. cloacae</i>	1	1 (100) ^c	0
<i>E. aerogenes</i>	1	0 (0)	0
<i>Morganella morganii</i>	1	0 (0)	0

^a EtG and EtS (1 mg/L of each) were added to fresh UTI specimens, which were then stored in sealed plastic vials (without preservative) at 22 °C for 1–5 days, after which EtG and EtS were measured by LC-MS.

^b Bacterial count, 10^3 to $>10^5$ CFU/mL.

^c Bacterial count, $>10^5$ CFU/mL.

Medichem Diagnostics), respectively. The EtG and EtS concentrations in unknown samples were calculated from peak-area ratios to the internal standard by reference to a calibration curve (single determinations). The detection and quantification limits were ~ 0.05 mg/L and ~ 0.1 mg/L, respectively, for both compounds (3).

The different pathogens identified among the 46 clinical urine specimens included in this study are listed in Table 1. *E. coli* was the predominant bacterial species, and it is also the primary causative agent of UTI ($\sim 80\%$ of cases), although other uropathogens, such as *Staphylococcus* (10%–15%), *Klebsiella*, *Enterobacter*, *Enterococcus*, *Proteus*, and *Pseudomonas* species infrequently cause disease (13). For both EtG and EtS, 9 urine specimens were found positive before supplementation (range, 0.3–39.3 mg/L), and for only EtG (0.2 mg/L), 1 was found positive.

In the majority (68%) of the urine specimens containing *E. coli*, a marked decrease in the EtG concentration was observed over time after storage at 22 °C, but not at 4 °C or –20 °C (see examples in Fig. 1). These results agree with those of previous studies, showing that most, but not all, *E. coli* strains possess β -glucuronidase activity (14–16). In 3 specimens, complete hydrolysis of the added EtG (1 mg/L) was noted after 24 h of storage at 22 °C. In the 2 specimens that contained the highest EtG concentrations, 37.3 and 39.3 mg/L before supplementation, the EtG concentrations were decreased to 11.2 and 0.7 mg/L, respectively, after storage for 5 days at 22 °C.

In 1 of 3 urine specimens containing *Klebsiella pneumoniae* and the single specimen containing *Enterobacter cloacae*, a gradual disappearance of EtG was also observed after storage at 22 °C, but the rate of EtG hydrolysis was

slower than for most specimens with *E. coli* tested under the same conditions (data not shown). These observations are consistent with previous results, which indicate that a few other pathogens causing UTI, including some strains of *Klebsiella* and *Enterobacter*, also possess low β -glucuronidase activity (15, 16).

To determine whether chemical preservatives could prevent bacterial hydrolysis of EtG, we added 1 mg/L each of EtG and EtS to 8 UTI urine specimens confirmed positive for *E. coli* and incubated them in tubes containing sodium fluoride (10 mg NaF/mL of urine) or without preservatives. In 6 of these specimens, marked or complete hydrolysis of EtG was observed in the tubes without preservatives after storage for 5 days at 22 °C, whereas EtG was found to be stable on storage in the tubes containing NaF.

No decrease in the EtS concentration on storage was observed in any of the UTI specimens examined in this study (Table 1). Likewise, 2 commercial preparations of sulfatase (type H1 from *Helix pomatia* and type VI from *Aerobacter aerogenes*; Sigma-Aldrich) were found not to hydrolyze EtS in urine samples.

EtG (4, 17, 18) and, more recently, EtS (3, 19, 20) have been introduced as sensitive and specific biomarkers for detection of recent alcohol consumption, with major advantages compared with conventional ethanol testing, including a much longer detection time (i.e., improved sensitivity) with retained high specificity. Our results demonstrated, however, that EtG may not be stable on storage if the urine specimens taken for analysis are infected with pathogens possessing β -glucuronidase activity (typically *E. coli*). In contrast, EtS was indicated to be completely stable to bacterial hydrolysis. The disappearance rate of EtG was temperature dependent, and refrigeration or freezing of samples, or use of sampling tubes containing fluoride preservatives, was effective in preventing, or markedly reducing, hydrolysis.

In some urine specimens, the tested EtG concentration (1 mg/L) was no longer detectable after storage at room temperature for 1 day. This concentration is in the range commonly observed in clinical practice (21), where $\sim 40\%$ of all EtG-positive urine samples collected for testing of recent alcohol consumption showed values < 10 mg/L. An even more apparent disappearance of EtG was noted in the 2 urine specimens containing high concentrations before supplementation (37.3 and 39.3 mg/L), in which much or most of the EtG had been hydrolyzed after storage for 5 days at room temperature. Accordingly, if biological specimens are stored under appropriate conditions (e.g., room temperature) or transported or mailed to the laboratory without refrigeration, freezing, or use of preservatives, the possibility of false-negative and falsely low EtG results always has to be considered. Even if precautions are taken to prevent bacterial degradation of EtG in the collected specimens, hydrolysis could possibly have taken place before sampling, e.g., in urine retained in the bladder during sleep of patients with UTIs or other pathologic conditions (22) or between time of death and autopsy in postmortem cases.

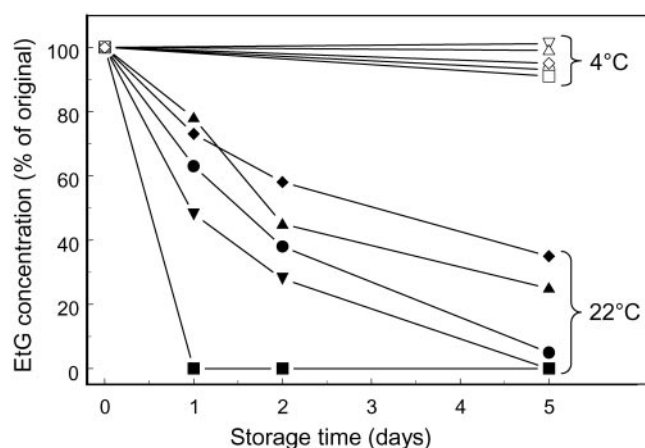


Fig. 1. Hydrolysis of EtG by bacterial β -glucuronidase in clinical urine specimens containing *E. coli*.

Fresh clinical urine specimens with confirmed bacterial growth (UTIs) were supplemented with EtG and EtS (1 mg/L of each substance) and split into tubes without preservatives that were placed at 4 °C (○, ◇, ▽, △, and □) and 22 °C (●, ◆, ▼, ▲, and ■). After 1 to 5 days of storage, samples were taken for analysis of EtG and EtS by LC-MS. Shown are the EtG results for 5 representative UTI specimens positive for *E. coli*.

In summary, the results of the present study demonstrated that EtG, but not EtS, is sensitive to bacterial hydrolysis, particularly when specimens are infected by *E. coli*, which is the most common source of UTIs. Given that UTIs caused by *E. coli* are among the most frequent bacterial infections encountered in clinical practice (13), this represents an obvious risk factor for false-negative and falsely low EtG results. Because EtG and EtS show similar windows of detection after alcohol consumption (3), it may therefore be advantageous and recommended to measure EtS instead of EtG for detection or confirmation of recent drinking, or at least to combine EtG with EtS analysis, which is possible by LC-MS (3, 23). In any case, ensuring that specimens are refrigerated or frozen and/or that sampling tubes contain fluoride preservatives to prevent bacterial growth is recommended practice.

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Parallel Assessment of Circulatory Fetal DNA and Corticotropin-Releasing Hormone mRNA in Early- and Late-Onset Preeclampsia, Xiao Yan Zhong,¹ Stefan Gebhardt,² Renate Hillermann,³ Kashafa Carelse Tofa,² Wolfgang Holzgreve,¹ and Sinuhe Hahn^{1*} (¹ University Women's Hospital/Department of Research, University Hospital Basel, Basel, Switzerland; Departments of ²Obstetrics and Gynecology and ³Genetics, University of Stellenbosch, Stellenbosch, South Africa; * address correspondence to this author at: Laboratory for Prenatal Medicine, University Women's Hospital/Department of Research, University Hospital Basel, Spitalstrasse 21, CH 4031 Basel, Switzerland; fax 41-61-265-9399, e-mail shahn@uhbs.ch)

Preeclampsia, a severe disorder of human pregnancy of unknown etiology, remains a major cause of fetal and maternal mortality (1, 2). Because of the heterogeneous nature of this disorder, it has been suggested that preeclampsia could be subclassified into two distinct forms to better understand the underlying causes (3). These two forms, termed early and late onset, are defined as the development of symptoms before or after 34 weeks of pregnancy, respectively (3). Results of several studies have indicated that the early-onset form is more severe, frequently leading to the delivery of growth-retarded premature babies, whereas the late-onset form is more evanescent and clinically of lesser importance (4).

Previous studies have indicated that cell-free fetal DNA and mRNA concentrations are increased in preeclampsia (5–8). Because these studies focused on either cell-free DNA or RNA alone, we examined the concentrations of these nucleic acids simultaneously in cases with early- and late-onset preeclampsia.

Approval for this study was granted by the respective Institutional Review Boards (Basel and Stellenbosch), and export of biological material was approved by the South African Department of Health. Written informed consent was obtained in all instances. In our study, maternal blood samples were collected from 37 pregnant women with manifest preeclampsia. Preeclampsia was defined by new-onset blood pressure of at least 140/90 mmHg in 2 determinations 4 h apart or by a diastolic blood pressure >110 mmHg, as well as an associated proteinuria of at least