Papers in Press. Published August 23, 2007 as doi:10.1373/clinchem.2007.089482
The latest version is at http://www.clinchem.org/cgi/doi/10.1373/clinchem.2007.089482

Postcollection Synthesis of Ethyl Glucuronide by Bacteria in Urine May Cause False Identification of Alcohol Consumption

(Department of Clinical Neuroscience, Karolinska Institute and Karolinska 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@ki.se) *Anders Helander,* Ingrid Olsson, and Helen Dahl*

Background: **Ethyl glucuronide (EtG) is a minor ethanol metabolite used as a specific marker to document recent alcohol consumption; confirm abstinence in treatment programs, workplaces, and schools; and provide legal proof of drinking. This study examined if bacterial pathogens in urine may enable postsampling synthesis of EtG and ethyl sulfate (EtS) from ethanol, leading to clinical false-positive results.**

Methods: **Urine specimens with confirmed growth of *Escherichia coli*, *Klebsiella pneumoniae*, or *Enterobacter cloacae* were stored at room temperature in the presence of ethanol. Ethanol was either added to the samples or generated by inoculation with the fermenting yeast species *Candida albicans* and glucose as substrate. EtG and EtS were measured by LC-MS.**

Results: **High concentrations of EtG (24-h range 0.5–17.6 mg/L) were produced during storage in 35% of *E. coli*-infected urines containing ethanol. In some specimens that were initially EtG positive because of recent alcohol consumption, EtG was also sensitive to degradation by bacterial hydrolysis. In contrast, EtS was completely stable under these conditions.**

Conclusions: **The presence of EtG in urine is not a unique indicator of recent drinking, but might originate from postcollection synthesis if specimens are infected with *E. coli* and contain ethanol. Given the associated risks for false identification of alcohol consumption and false-negative EtG results due to bacterial degradation, we recommend that measurement of EtG be combined with EtS, or in the future possibly replaced by EtS.**

Early recognition of problem drinking or relapse is important to ensure adequate alcohol treatment strategies *(1)*. This goal has been hampered by a lack of sufficiently sensitive and specific diagnostic methods. The reliability of self-reporting is limited by denial and underreporting *(2)*. The time frame for identifying alcohol use by ethanol testing is usually limited to <12 h, because of rapid metabolism and excretion *(3)*. Research has therefore focused on developing alcohol biomarkers with a longer detection window *(4)*.

A new laboratory marker for detecting recent alcohol consumption is ethyl glucuronide (EtG) *(5)*. EtG and ethyl sulfate (EtS) *(6)* are minor ethanol metabolites formed by uridine diphosphate–glucuronosyltransferase (UGT) and sulfotransferase (SULT), respectively, and excreted in urine for a longer time than ethanol *(7–10)*. Positive EtG and/or EtS test results thus provide a strong indication that the person has recently consumed alcohol, even when ethanol is no longer detectable *(9)*. LC-MS methods are available for EtG and EtS detection *(6, 10)*, as is an enzyme immunoassay for EtG (DRI® EtG, Microgenics).

EtG has been recommended for forensic application *(11–13)* and is used for documentation of abstinence in treatment programs, for alcohol testing in the workplace and schools, and as legal proof of drinking (known as "the 80-h alcohol test"). However, the high diagnostic sensitivity of the EtG test has also produced adverse publicity *(14)*, because unintentional ethanol intake from ethanol-based mouthwash *(15)* and hand sanitizers *(16)* may also generate positive results. The United States Substance Abuse and Mental Health Services Administration recently warned against using a positive EtG as primary or sole evidence of drinking for disciplinary and legal action *(17)*.

Bacterial contamination of urine may cause false-negative EtG test results *(18)*. Many strains of *Escherichia coli*, the main source of urinary tract infections, contain the enzyme β-glucuronidase, which hydrolyzes EtG. Given that UGT and SULT activity also occur with some bacteria *(19, 20)*, we examined whether human pathogens may enable postcollection synthesis of EtG and EtS from ethanol in urine.

Fresh human urine specimens (anonymous surplus volumes) with confirmed growth of common pathogenic bacteria (*E. coli*, n = 36; *Klebsiella pneumoniae*, n = 6; *Enterobacter cloacae*, n = 6), as identified by culture on standard solid media, were used (study approved by the local ethics committee). The samples had been submitted for routine diagnostic testing in the Department of Clinical Microbiology, Karolinska University Hospital, and were stored refrigerated until use.

In the 1st experiment we added ethanol (final concentration 1.0 g/L) to urine samples and split them into tubes that were capped and stored at 4 °C and 22 °C. The same samples without addition of ethanol, or supplemented with ethanol and 10 g/L sodium fluoride as preservative, and uninfected urines served as controls. In the 2nd experiment ethanol was generated in the urine samples by inoculation with the fermenting yeast species *Candida albicans* (1 000 000 colony-forming units/L) and 20 g/L glucose as substrate. At the start of the experiment, and after different storage times at 4 °C and 22 °C, aliquots were stored at −20 °C before analysis of EtG, EtS, and ethanol.

EtG and EtS were quantified by an LC-MS method *(6, 9, 10)*. Analysis was performed in the negative-ion mode using selected ion monitoring of the deprotonated ions at *m/z* 125 for EtS and *m/z* 130 for EtS-D5, and at *m/z* 221 and *m/z* 226 for EtG and EtG-D5. We purchased EtS from TCI and EtG and EtG-D5 from Medichem Diagnostics. EtS-D5 was synthesized *(9)*. The previously determined detection limit was 0.1 mg/L; the routine clinical cutoff used in our laboratory is 0.5 mg/L. All positive EtG results by LC-MS were confirmed by LC-tandem MS

(Perkin–Elmer 200 LC 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 interference by ion suppression was noted.

The ethanol concentration was determined enzymatically using alcohol dehydrogenase on a Hitachi 917 analyzer.

Of the 36 urine specimens infected by *E. coli*, 10 were positive for EtG (range 2.6–135.9 mg/L, mean 25.7 mg/L, median 10.4 mg/L) and EtS (range 1.3–20.0 mg/L, mean 5.0 mg/L, median 3.5 mg/L) at the start of the experiment, indicating that these patients had recently consumed alcohol. After these 10 samples were stored for 5 days at 22 °C, EtG was no longer detectable in 5 (50%), whereas the EtS concentrations remained unchanged. A disappearance of EtG, but not of EtS, was also observed after the samples had been supplemented with ethanol (Table 1). In 3 samples that initially contained 11.7–46.6 mg/L EtG, the concentrations were below the routine clinical cutoff (<0.5 mg/L) after 24 h storage at 22 °C. These samples also showed a gradual disappearance of EtG at 4 °C, albeit at a much slower rate, whereas sodium fluoride was effective in preventing EtG degradation both at 4 °C and 22 °C (data not shown).

In 9 (35%) of the 26 urine specimens with confirmed growth of *E. coli* that were initially negative for EtG and EtS, formation of EtG but not of EtS was observed with time at 22 °C after addition of 1 g/L ethanol. In 7 samples (Fig. 1A), EtG concentrations above the clinical cutoff were observed after 24-h storage (range 0.5–17.6 mg/L, mean 5.2 mg/L, median 3.3 mg/L), and after 5 days the concentrations ranged from 0.3 to 35.2 mg/L (mean 8.9 mg/L, median 2.4 mg/L, n = 9). Slow formation of EtG was also observed in 3 samples in the presence of added sodium fluoride and in 2 samples stored at 4 °C. After addition of ethanol to 1 urine specimen that initially contained 8.9 mg/L EtG and 2.0 mg/L EtS, the EtG concentration first increased to 17.6 mg/L after 24-h storage but then decreased to 5.6 mg/L after 48 h and then

Table 1. Stability of the minor ethanol metabolites EtG and EtS during storage of infected urine specimens.

Test conditions	Uropathogen identified		
	E. coli, n	*K. pneumoniae*, n	*E. cloacae*, n
Infected urine specimens incubated with 1.0 g/L ethanol for 5 days[a]	36	6	6
Specimens initially positive for EtG and EtS[b]	10	1	4
EtG unchanged after 5 days	5	6	6
EtG negative or decreased after 5 days	5	0	0
EtS unchanged after 5 days	10	6	6
EtS negative or decreased after 5 days	0	0	0
Specimens initially negative for EtG and EtS	26	5	2
EtG negative after 5 days	17	5	2
EtG positive after 5 days[c]	9	0	0
EtS negative after 5 days	26	5	2
EtS positive after 5 days	0	0	0

[a] Ethanol was added to fresh urine specimens with confirmed growth of *E. coli*, *K. pneumoniae*, or *E. cloacae* and stored in sealed plastic vials without preservative at 22 °C for 5 days.

[b] Samples initially positive for EtG and EtS indicated that these patients had recently consumed alcohol. EtG and EtS were measured by LC-MS and the detection limit was approximately 0.1 mg/L for both compounds.

[c] All positive LC-MS results were confirmed positive by liquid chromatography-tandem mass spectrometry.

[d] n, Number of specimens.

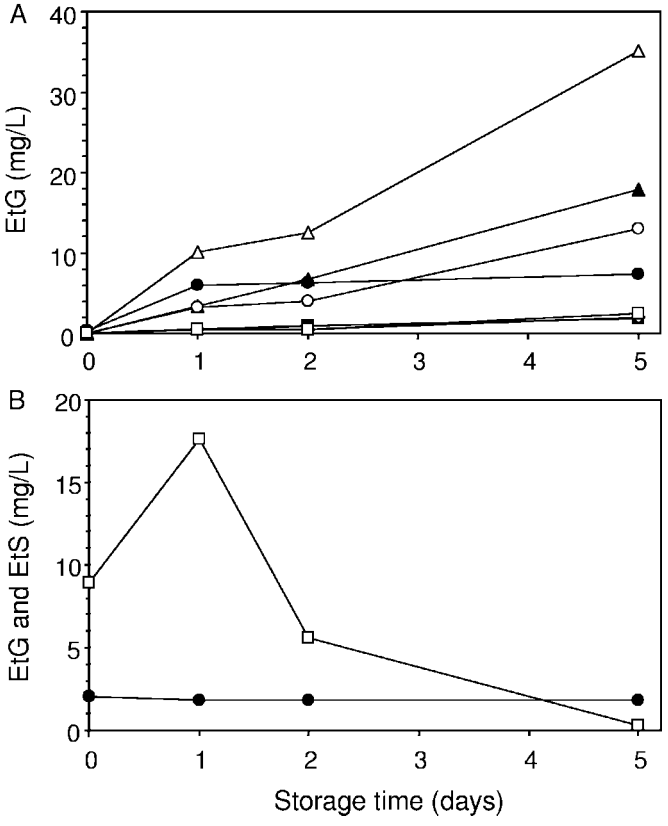

Fig. 1. Formation of the ethanol metabolite EtG in *E. coli* infected urine samples after addition of ethanol.

(*A*), urine specimens with confirmed growth of *E. coli* were supplemented with 1.0 g/L ethanol and stored at 22 °C. Individual EtG results for 7 urine samples are indicated by different symbols (the data for 3 samples that produced low concentrations of EtG partly overlap). (*B*), results for 1 urine specimen with confirmed growth of *E. coli* that showed both synthesis and degradation of EtG. The sample was initially positive for EtG (□) and EtS (●) and showed variable EtG but unchanged EtS concentrations with time after addition of ethanol and storage at 22 °C.

to <0.5 mg/L after 5 days (Fig. 1B). The corresponding EtS concentrations were stable at all times.

After 7 urine specimens containing *E. coli* and 5 uninfected control urines were supplemented with *C. albicans* and glucose to generate ethanol (all samples were initially negative for ethanol), the ethanol concentrations after 7-day storage at 22 °C ranged from 0.73 to 1.47 g/L (median 1.17 g/L). Formation of EtG (range 1.8–71.4 mg/L) was observed in 3 specimens containing *E. coli*, but in none of the uninfected controls. No formation of EtS was detected in these experiments.

No disappearance or formation of EtG or EtS was observed in the 12 urine specimens with confirmed growth of *K. pneumoniae* or *E. cloacae* after addition of ethanol or *C. albicans* and glucose and storage at 22 °C for 5 days (Table 1).

EtG has been considered specific for alcohol consumption and detectable only after in vivo ethanol metabolism, and hence EtG testing is used as a basis for disciplinary and legal action *(17)* and in forensic autopsy cases *(13)*. A recent debate relates to the excellent analytical sensitivity of this test that, in combination with a low clinical cutoff concentration, may cause positive results attributable to unintentional ethanol exposure *(14–16)*. To the best of our knowledge, no true false-positive EtG result has been reported without such exposure. Nonetheless, the present study demonstrated that EtG could be formed in a biological specimen after collection, if the specimen is infected with *E. coli* and ethanol is present or produced during storage. In our tested samples the formation of EtG was rapid and was not always prevented by addition of sodium fluoride or storage at refrigerator temperature.

Bacterial and fungal infections are common in clinical practice, with *E. coli* being the primary pathogen responsible for urinary tract infections. Ethanol may be formed in unpreserved biological specimens because of microbial contamination and fermentation, and this risk is especially high in diabetic patients as a result of glycosuria. Accordingly, considering the potential serious disciplinary and legal consequences if an individual is falsely accused of alcohol consumption on the basis of an incorrect EtG result, caution is advised when interpreting EtG test results, and the risk for postcollection ethanol formation must be considered.

The results of our study also confirm previous observations that EtG is sensitive to bacterial hydrolysis, but EtS is not *(18)*. Accordingly, in situations in which EtG-positive urine is infected from the start, or becomes contaminated during handling, there is a risk for false-negative results and alcohol use will remain undetected.

The lack of EtS formation or degradation detected under test conditions and the similar detection windows and sensitivities for recent alcohol consumption observed for the unique ethanol metabolites EtG and EtS *(9)* indicate that EtS testing should accompany, be used to verify, or in the future possibly replace EtG testing. The results further indicate that EtS is a more suitable test than EtG to distinguish antemortem ingestion of ethanol from postmortem synthesis in forensic toxicological analysis *(13)*. Mass spectrometric methods can easily be modified to also quantify EtS *(6, 9)*. If the analysis initially focuses solely on EtG, EtS may be introduced as a verification assay. However, a negative EtG screening result will usually not be followed up with confirmatory analysis, and drinking will thereby remain undetected.

Grants/funding support: The present work was funded by the Karolinska Institute and the Stockholm County Council, Stockholm, Sweden.
Financial disclosures: None declared.

References

1. Babor TF, Aguirre-Molina M, Marlatt GA, Clayton R. Managing alcohol problems and risky drinking. Am J Health Promot 1999;14:98–103.
2. Fuller RK, Lee KK, Gordis E. Validity of self-report in alcoholism research: results of a Veterans Administration cooperative study. Alcohol Clin Exp Res 1988;12:201–5.
3. Helander A, Beck O, Jones AW. Laboratory testing for recent alcohol consumption: comparison of ethanol, methanol, and 5-hydroxytryptophol. Clin Chem 1996;42:618–24.
4. Helander A. Biological markers in alcoholism. J Neural Transm Suppl 2003;15–32.
5. Seidl S, Wurst FM, Alt A. Ethyl glucuronide: a biological marker for recent alcohol consumption. Addict Biol 2001;6:205–12.
6. Helander A, Beck O. Mass spectrometric identification of ethyl sulfate as an ethanol metabolite in humans. Clin Chem 2004;50:936–7.
7. Schmitt G, Aderjan R, Keller T, Wu M. Ethyl glucuronide: an unusual ethanol metabolite in humans. Synthesis, analytical data, and determination in serum and urine. J Anal Toxicol 1995;19:91–4.
8. Dahl H, Stephanson N, Beck O, Helander A. Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide. J Anal Toxicol 2002;26: 201–4.
9. Helander A, Beck O. Ethyl sulfate: a metabolite of ethanol in humans and a potential biomarker of acute alcohol intake. J Anal Toxicol 2005;29: 270–4.
10. Stephanson N, Dahl H, Helander A, Beck O. Direct quantification of ethyl glucuronide in clinical urine samples by liquid chromatography-mass spectrometry. Ther Drug Monit 2002;24:645–51.
11. Wurst FM, Skipper GE, Weinmann W. Ethyl glucuronide: the direct ethanol metabolite on the threshold from science to routine use. Addiction 2003; 98(Suppl 2):51–61.
12. Skipper GE, Weinmann W, Thierauf A, Schaefer P, Wiesbeck G, Allen JP, et al. Ethyl glucuronide: a biomarker to identify alcohol use by health professionals recovering from substance use disorders. Alcohol Alcohol 2004;39: 445–9.
13. Kugelberg FC, Jones AW. Interpreting results of ethanol analysis in postmortem specimens: a review of the literature. Forensic Sci Int 2007;165:10–29.
14. Helliker K. A test for alcohol and its flaws: a new screen detects Sunday's gin in Monday's urine but it may be ensnaring some innocent people too. Beer or hand sanitizer? Wall St J 2006 Aug 12;:.
15. Costantino A, Digregorio EJ, Korn W, Spayd S, Rieders F. The effect of the use of mouthwash on ethylglucuronide concentrations in urine. J Anal Toxicol 2006;30:659–62.
16. Rohrig TP, Huber C, Goodson L, Ross W. Detection of ethylglucuronide in urine following the application of Germ-X. J Anal Toxicol 2006;30:703–4.
17. Center for Substance Abuse Treatment. The role of biomarkers in the treatment of alcohol use disorders. Substance Abuse Treatment Advisory 2006;5:1–8.
18. Helander A, Dahl H. Urinary tract infection: a risk factor for false-negative urinary ethyl glucuronide but not ethyl sulfate in the detection of recent alcohol consumption. Clin Chem 2005;51:1728–30.
19. Bock KW. Vertebrate UDP-glucuronosyltransferases: functional and evolutionary aspects. Biochem Pharmacol 2003;66:691–6.
20. Chen WT, Liu MC, Yang YS. Fluorometric assay for alcohol sulfotransferase. Anal Biochem 2005;339:54–60.

Previously published online at DOI: 10.1373/clinchem.2007.089482