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Ethyl glucuronide, a new biochemical marker for acute alcohol intake

Studies on possible causes for false-negative
or false-positive results

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♥ *To my parents*

ABSTRACT

In recent years, there has been a growing interest in various biochemical markers for detecting acute and chronic alcohol consumption. Biochemical markers for acute and chronic drinking play important roles in detecting alcohol use, abuse and dependence in hospital settings, work place settings, traffic medicine and in forensic toxicology examinations. The alcohol biomarkers can be distinguished into two main classes: 1) tests that are sensitive enough to detect a single intake of alcohol, such as ethanol, 5-hydroxytryptophol (5HTOL), ethyl glucuronide (EtG) and ethyl sulfate (EtS), and 2) tests that are able to detect chronic heavy drinking, or indicate body organ or tissue damage caused by long-term exposure to alcohol, such as carbohydrate-deficient transferrin (CDT), γ -glutamyl transferase (GGT), aspartate and alanine aminotransferase (AST and ALT), and the mean corpuscular volume of erythrocytes (MCV).

After consumption of alcoholic beverages, a small portion (<5%) of the ethanol is excreted unchanged in the urine, sweat and breath. The major part (>95%) instead becomes metabolized mainly in the liver in a two-stage oxidation process, first to acetaldehyde by alcohol dehydrogenase and further to acetate by aldehyde dehydrogenase. Another very small part undergoes non-oxidative metabolism to produce the phase II products EtG and EtS. The interest in EtG and EtS as biochemical markers for acute alcohol intake has primarily focused on the observation that the washout rates for these direct ethanol metabolites are much slower than for the parent compound, allowing for a longer detection time (i.e., higher sensitivity). A positive finding of EtG and/or EtS in urine or serum thus provides a strong indication that the person was recently drinking alcohol, even if drinking is denied and also for some time after ethanol itself is no longer detectable.

The purpose of this thesis was to evaluate the accuracy of EtG in urine as a biochemical marker for acute alcohol consumption, by studying possible sources of error that can affect the result of the analysis and cause false-negative or false-positive results. Urinary EtG was determined by liquid chromatography-mass spectrometry (LC-MS).

The first study demonstrated that glucuronide conjugation of ethanol forming EtG represents a minor elimination pathway (<0.03%) in the human body, and also confirmed that EtG remains detectable in the urine for many hours after the ethanol has been eliminated. Drinking large amount of fluid prior to voiding was found sufficient to markedly lower the urinary concentration of EtG, but this practice did not influence the concentration of ethanol or the EtG/creatinine ratio. Expressing EtG as a ratio to creatinine may thus be recommended in clinical practice, to compensate for urine dilution. In the second study, no significant accumulation of EtG or 5HTOL was observed, upon multiple-dose administration of ethanol at 0.8 g/kg/day for one week to healthy volunteers. Moreover, the detection time in urine for EtG was demonstrated to be longer than for 5HTOL. In the last study, it was found that EtG but not EtS is sensitive to bacterial hydrolysis, particularly in specimens infected by *Escherichia coli*, which is a well known source of β -glucuronidase. Accordingly, to reduce the risk for obtaining falsely low or false-negative EtG results, specimens should be stored refrigerated or frozen prior to analysis. Sampling urine in test tubes containing sodium fluoride was also found effective to prevent bacterial hydrolysis of EtG.

In conclusion, the present results demonstrated that urinary EtG is a very sensitive and specific biochemical marker for acute alcohol intake. Urine dilution and bacterial hydrolysis were identified as possible causes for falsely low or false-negative EtG results. For routine clinical use, it may therefore be recommended to express urinary EtG as a ratio to creatinine, and to combine EtG and EtS analysis, which is possible by LC-MS.

LIST OF ORIGINAL PAPERS

This thesis is based upon the following papers, which are referred to in the text by their Roman numerals.

- I. Dahl H, Stephanson N, Beck O and Helander A. (2002). Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide. *Journal of Analytical Toxicology*. 26, 201-4.

- II. Sarkola T, Dahl H, Eriksson CJP and Helander A. (2003). Urinary ethyl glucuronide and 5-hydroxytryptophol levels during repeated ethanol ingestion in healthy human subjects. *Alcohol and Alcoholism*. 38, 347-51.

- III. Helander A and Dahl H. (2005). Urinary tract infection: a risk factor for false-negative urinary ethyl glucuronide but not ethyl sulfate in the detection of recent alcohol consumption. *Clinical Chemistry*. 51, 1728-30.

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ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUDIT	Alcohol Use Disorders Identification Test
CAGE	Cutting down, Annoyance by criticism, Guilty feeling, Eye opener
CDT	Carbohydrate-deficient transferrin
Crea	Creatinine
<i>E. coli</i>	<i>Escherichia coli</i>
EtG	Ethyl glucuronide
EtS	Ethyl sulfate
FAEE	Fatty acid ethyl esters
GC-MS	Gas chromatography-mass spectrometry
GGT	γ -Glutamyl transferase
HPLC	High-performance liquid chromatography
5HIAA	5-Hydroxyindole-3-acetic acid
5HTOL	5-Hydroxytryptophol
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MCV	Mean corpuscular volume of erythrocytes
Mm-MAST	Malmö-modified Michigan Alcoholism Screening Test
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
UDPGA	Uridine-5-diphospho- β -glucuronic acid
UGT	UDP-glucuronosyltransferase
UTI	Urinary tract infection

INTRODUCTION

In Sweden, about 90% of the adult population use alcohol more or less regularly, and at least 500.000 people drink alcohol in harmful amounts. It has been estimated that 10–15% of all Swedish men and 3–5% of the women are at risk of having serious alcohol problems, and many die from sickness or injury caused by alcohol. The use and abuse of alcohol cost the Swedish society enormous amounts each year, not only related to increased health care costs but to factors such as lost productivity (Johnson, 2003).

In a Swedish study carried out in a surgical emergency ward (Forsberg et al., 2000, 2003), about 15% of the patients screened positive for hazardous alcohol consumption. It was also demonstrated that brief assessment with feedback regarding riskful alcohol consumption could be a useful way to reduce harmful drinking. An elevated, harmful level of alcohol consumption may lead to higher rates of sickness absence, and the associated hangover symptoms also increase the risk of causing and getting involved in accidents (Wiese et al., 2000). A Swedish workplace study indicated that individuals with moderately elevated or harmful levels of alcohol consumption show an increase in sick-days (Hermansson et al., 2002). These results should encourage workplaces to use alcohol screening, for example during routine health examinations at the company health care service.

Collecting information about a person drinking habits is usually a difficult task. Screening for alcohol abuse can be performed by interview, or by using different alcohol questionnaires such as the AUDIT (Allen et al., 1997), CAGE (Mayfield et al., 1974) and Mm-MAST (Kristenson et al., 1985). However, it is well known that people may not always report their alcohol intake correctly, but often deny drinking or underreport the true amount (Midanik et al., 1989; Helander et al., 1999). For this reason, harmful drinking and alcohol abuse are probably much under-diagnosed. Accordingly, there is a need to find more objective methods to identify persons with elevated and harmful drinking levels in the first place, and to confirm abstinence from alcohol or detect relapse during outpatient treatment of those diagnosed with having alcohol-related problems.

The most obvious objective way to confirm alcohol consumption is to test for the presence of alcohol (ethanol) in breath or body fluids. A problem with alcohol testing is that the ethanol is rapidly cleared from the body, and this method therefore suffers from low sensitivity. To try to overcome this problem, various laboratory tests, or biochemical markers, for alcohol use and abuse have been suggested over the years. However, because many of these biochemical markers are indirectly associated with alcohol consumption, they have

sometimes shown a low specificity for alcohol and/or a low sensitivity for harmful drinking, causing problems when introduced into clinical practice. Accordingly, there is a need and continuous search for new and improved alcohol markers.

ETHANOL METABOLISM

Ethanol is a small, polar molecule with a low molecular weight and weak charge, which facilitates penetration through biological membranes. After alcohol ingestion, the ethanol is rapidly absorbed from the stomach (~20%) and intestines (~80%) (Jones, 1990). The absorption becomes delayed if there is food in the stomach (Jones, 1996). Ethanol then distributes in total body water, which averages about 75% of the body mass for men and 66% for women, but the blood ethanol concentration obtained after drinking alcohol also depends on factors such as the body weight and fat content.

Less than 5% of the ingested ethanol is excreted unchanged in urine, breath and sweat, while the major part (>95%) is eliminated mainly in the liver in a two-stage oxidation process at a rate of about 0.1 g/kg/h (Jones, 1996) (Figure 1). The oxidation of ethanol to form acetaldehyde takes place in the cytoplasm of the hepatocytes by the action of NAD⁺-dependent alcohol dehydrogenase (ADH), and the acetaldehyde is further oxidized to acetate by NAD⁺-dependent aldehyde dehydrogenase (ALDH) in the mitochondria. The acetate enters the circulation and eventually forms carbon dioxide and water by oxidation in the peripheral tissues. Furthermore, a small amount of ethanol becomes oxidized to acetaldehyde via CYP2E1, belonging to the cytochrome P450 family, or by catalase in the microsomal ethanol oxidizing system (MEOS), but these systems appear to be less important for the overall elimination of ethanol.

Another very small part of the ethanol undergoes non-oxidative metabolism to produce end products such as fatty acid ethyl esters (FAEE) via FAEE synthase, phosphatidylethanol (PEth) via phospholipase D, ethyl glucuronide (EtG) via UDP-glucuronosyltransferase (UGT) and ethyl sulfate (EtS) via sulfotransferase (SULT) (Figure 1).

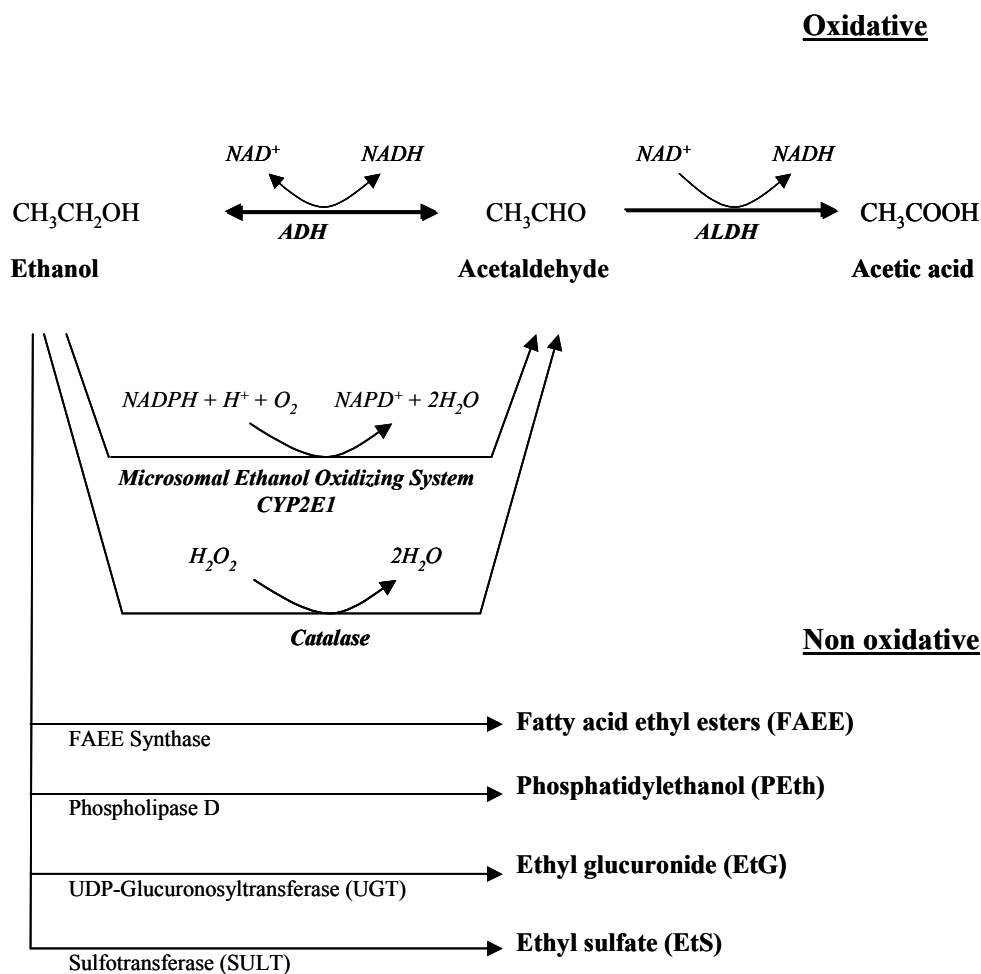


Figure 1. The major oxidative pathway and the minor non-oxidative pathways for ethanol elimination in the human body.

BIOCHEMICAL ALCOHOL MARKERS

The need for improved biochemical markers to be used for detection of sustained, heavy alcohol consumption but also of recent drinking has been widely recognized (Allen and Litten, 2001; Helander and Eriksson, 2002; Conigrave et al., 2002). The interest in and knowledge of alcohol biomarkers has also grown rapidly in recent years and biochemical markers have been demonstrated to play important clinical roles in detecting persons with harmful drinking levels or alcohol abuse in hospital settings (Spies and Rommelspacher, 1999), workplace settings (Hermansson et al., 2000), traffic medicine (Voas et al., 2000) and forensic toxicology (Jones et al., 2000). The biochemical alcohol markers are often distinguished into the following main classes:

1. Markers of acute alcohol consumption are laboratory tests sensitive enough to detect a single intake of alcohol within the previous ~24–48 h, and include tests such as ethanol, 5-hydroxytryptophol (5HTOL) (Voltaire et al., 1992; Helander et al., 1994), EtG (Schmitt et al., 1997; Wurst et al., 1999) and EtS (Helander and Beck, 2005).
2. Markers of chronic, heavy alcohol consumption are laboratory test that can detect sustained, harmful intake of alcohol, and include carbohydrate-deficient transferrin (CDT) (Stibler, 1991), PEth (Alling et al., 1984), and the mean corpuscular volume of erythrocytes (MCV) (Chick et al., 1981), but also tests to indicate the organ and tissue damage caused by long-term alcohol exposure, such as the liver enzymes γ -glutamyl transferase (GGT) (Chick et al., 1981) and aspartate and alanine aminotransferase (AST and ALT) (Conigrave et al., 1995).
3. Trait markers of alcohol dependence are tests aimed to indicate if a person has a genetic predisposition of becoming dependent on ethanol. Platelet monoamine oxidase (Sullivan et al., 1990) and adenylyl cyclase (Hellevuo et al., 1997) have, for example, been proposed as trait markers for alcohol dependence.

Sensitivity and Specificity

Much research has focused on developing new and more accurate biochemical markers for alcohol use and abuse. Biochemical markers are usually evaluated in terms of diagnostic sensitivity and specificity. The sensitivity and specificity for any biochemical marker depends on the cutoff, or threshold limit, set to distinguish between a normal and an abnormal value. The ideal marker should be both 100% sensitive and specific, although this is hardly ever achieved. A common problem when evaluating alcohol markers is also the difficulty to obtain correct information about drinking levels. The sensitivity and specificity for biochemical markers are calculated as follows:

➤ Sensitivity (%) = True positives / (True positives + False negatives)

➤ Specificity (%) = True negatives / (True negatives + False positives)

MARKERS OF ACUTE ALCOHOL CONSUMPTION

Ethyl Glucuronide (EtG)

EtG is a conjugated direct metabolite of ethanol that is formed by reaction with uridine-5-diphospho- β -glucuronic acid (UDPGA) which is catalyzed by the endoplasmic reticulum UDP-glucuronosyltransferase (UGT) enzymes (de Wildt et al., 1999; Tukey and Strassburg, 2001). The pathway of ethanol elimination via conjugation with UDPGA was first described in 1901 by Neubauer. In 1952, a dose-dependent formation of EtG, isolated as the tri-acetyl methyl ester, was demonstrated in rabbits, and 0.5–1.6% of the administered ethanol dose was excreted in the urine as EtG (Kamil et al., 1952). EtG was later determined also in human urine (Jaakonmaki et al., 1967; Kozu et al., 1973; Schmitt et al., 1995). In 1995, EtG was synthesized and identified in human serum (Schmitt et al., 1995).

The renewed interest in EtG, and then primarily related to its use as a biochemical marker for acute alcohol intake, originates from the observation that the washout rate for EtG is much slower than for the parent compound (Schmitt et al., 1997; Wurst et al., 1999), but also to the development of sensitive and specific analytical methods based on gas chromatographic-mass spectrometric (GC-MS) (Wurst et al., 1999) and liquid chromatographic-mass spectrometric (LC-MS) techniques (Stephanson et al., 2002). A positive finding of EtG in urine or serum thus provides a strong indication that the person was recently drinking alcohol, even in cases when the ethanol itself is no longer measurable. EtG was demonstrated to be eliminated with a half-life of ~2.5 h (Schmitt et al., 1997) and to show high inter-individual variation with a poor correlation with the concentration of ethanol (Bergström et al., 2003). The latter observation can at least partly be explained by the time-lag between the excretion profiles for ethanol and EtG.

The high sensitivity of EtG for recent drinking is evident from the observation that even intake of a very low dose (~7 g) of ethanol is detectable in the urine after 6 h (Stephanson et al., 2002). Depending on the amount of ethanol consumed, EtG has been reported to be measurable in the urine for up to 4 days after the last intake (Seidl et al., 2001). EtG can also be detected in samples of hair (Pragst et al., 2000; Skopp et al., 2000; Alt et al., 2000; Janda et al., 2002), other body fluids except blood and urine, and in tissue extracts (Wurst et al., 1999; Schloegl et al., 2006).

Ethyl Sulfate (EtS)

EtS is another conjugated, direct ethanol metabolite that is formed by reaction with sulfate by the action of cytosolic sulfotransferase (Vestermark and Boström, 1959; Boström and Vestermark, 1960; Bernstein et al., 1984; Manautou and Carlson, 1992). In humans, the elimination pathway for ethanol via sulfate conjugation to produce EtS was first established in 2004 by LC-MS (Helander and Beck, 2004). This study also demonstrated that only a very small fraction (<0.1%) of the ingested ethanol undergoes sulfate conjugation. Furthermore, these conjugated ethanol metabolites showed very similar urinary excretion profiles, but EtS was typically present in lower amounts compared with EtG. Recently there has been an increased focus on EtS as a biochemical marker for acute alcohol intake (Dresen et al., 2004; Helander and Beck, 2005).

Fatty Acid Ethyl Esters (FAEE)

FAEE are products of non-oxidative metabolism of ethanol via FAEE synthase (Laposata and Lange, 1986). FAEE have been proposed as a sensitive and specific biochemical marker of acute alcohol intake (Lange et al., 1983; Kinnunen et al., 1984). FAEE can be detected for up to at least 24 h after alcohol intake (Doyley et al., 1996), although a recent study reported a faster elimination of FAEE compared with earlier studies (Bisaga et al., 2005). FAEE may also be important in the pathogenesis of organ damage caused by alcohol abuse, since high FAEE concentrations were found in alcohol-related organ damage (Laposata, 1998).

5-Hydroxytryptophol (5HTOL)

During ethanol metabolism, there is an interaction with the metabolism of serotonin (5-hydroxytryptamine, 5HT) resulting in a shift from production of 5-hydroxyindole-3-acetic acid (5HIAA) towards formation of 5HTOL (Davis et al., 1967) (Figure 2). 5HTOL is normally the minor metabolite of 5HT (<1%) but its formation increases dramatically in a dose-dependent manner after alcohol consumption. At the same time the formation of the major metabolite 5HIAA becomes decreased (Helander et al., 1993). This metabolic shift from the predominant oxidative towards the reductive 5HT pathway occurs because of competitive inhibition of mitochondrial ALDH by acetaldehyde, and the increased cytosolic NADH/NAD⁺ ratio, both of which favor 5HTOL formation at the expense of 5HIAA (Walsh, 1973; Svensson et al., 1999). Furthermore, the reoxidation of 5HTOL to 5HIAL by ADH is inhibited during ethanol oxidation (Consalvi et al., 1986).

Soon after drinking alcohol, the urinary 5HTOL concentration becomes markedly increased and it will not return to baseline levels until several hours after the ethanol is no longer measurable (Helander et al., 1993). Based on this time-lag, urinary 5HTOL has been used clinically as a sensitive biochemical marker of recent alcohol consumption (Voltaire et al., 1992; Helander et al., 1994). To secure the specificity of the marker, 5HTOL should be expressed as a ratio to 5HIAA rather than to creatinine, because dietary serotonin (high amounts in banana and pineapple) might otherwise cause false-positive results. This practice also compensates for variations in the urinary concentration of 5HTOL caused by external and internal dilution of the urine (Helander et al., 1992). Gender or genetic variations in the ADH and ALDH isoenzyme patterns seemingly do not influence the baseline ratio of 5HTOL/5HIAA (Helander et al., 1994; Helander et al., 1996). The only known factor apart from alcohol intake that increases the urinary 5HTOL/5HIAA ratio is treatment with ALDH inhibitors such as disulfiram (Antabuse) and cyanamide (Dipsan) (Beck et al., 1995).

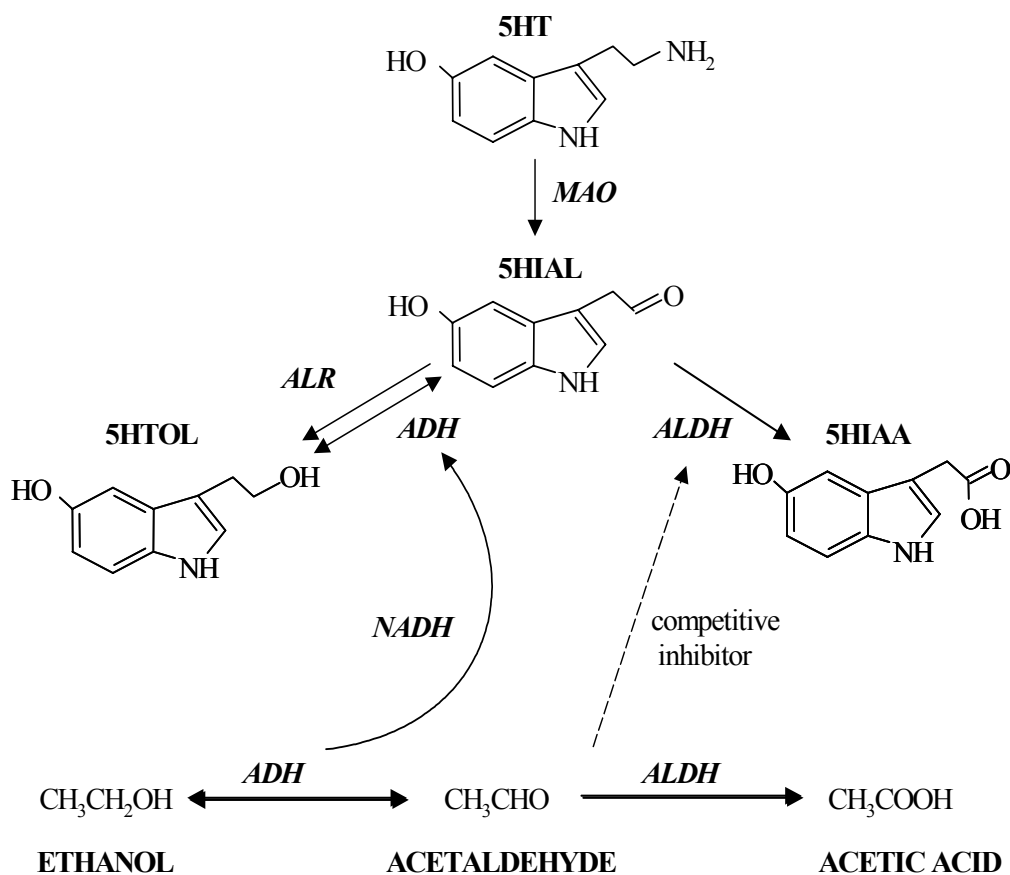


Figure 2. The interaction between serotonin (5HT) and ethanol metabolism.

Testing of urinary 5HTOL/5HIAA as a biochemical marker for acute alcohol intake has been applied in clinical practice to confirm abstinence and detect relapse drinking during treatment of alcohol-dependent subjects in outpatient settings (Voltaire Carlsson et al., 1993; Borg et al., 1995). Furthermore, the 5HTOL/5HIAA test has found uses in forensic medicine, because it can distinguish ingested from microbially formed ethanol, that might occur in postmortem specimens and in urine samples collected from diabetic patients with urinary tract infections (UTI) (Helander et al., 1992; Helander et al., 1995).

AIMS

General Aim

The purpose of this work was to evaluate the accuracy of urinary EtG as a biochemical marker for acute alcohol intake in clinical practice, by identifying possible sources of error that can interfere with the result of the analysis and cause false-negative or false-positive EtG results.

Specific Aims

- To compare the urinary excretion characteristics of EtG with that of ethanol in healthy human subjects, with focus on the effect of water-induced diuresis.

- To investigate the stability, reproducibility and any possible accumulation of EtG and 5HTOL in urine, after healthy human subjects ingested single and multiple oral doses of ethanol.

- To evaluate whether the presence of *Escherichia coli*, or any other common human pathogen, in urine specimens, resulting from urinary tract infections (UTI) or possibly contamination during sampling and handling, could give false-negative results for EtG and EtS due to hydrolysis by bacterial β -glucuronidase and sulfatase enzymes, and, if so, to try to identify methods that are able to prevent bacterial hydrolysis.

MATERIALS AND METHODS

Experimental Design

Study I

Six healthy volunteers (three women and three men), all being social drinkers, abstained from alcohol consumption for at least 48 h before starting the experiment, according to self-report. A light morning meal was allowed approximately 1.5 h before the subjects ingested an ethanol dose of 0.5 g/kg as beer (5%, v/v) in 30 min. At 3 h from the start of ethanol intake, they drank an equal volume of tap water. Urine sampling was performed immediately before starting the experiment and at timed intervals over 31.5 h. The samples were stored at -20°C until taken for analysis of ethanol using an enzymatic ADH method and for EtG by LC-MS (Stephanson et al., 2002).

Study II

Nine healthy female, all social drinkers, were told to avoid alcohol beverages for one week before entering the study and during the study period only the alcohol from the study was allowed. The subjects drank ethanol in juice (8%, w/v) or placebo (juice) in random order. The intervention consisted of 0.4 g/kg ethanol or placebo twice daily (morning and evening) over 8 consecutive days, starting in the evening of day 1. Urine sampling was performed every morning (first morning void) during the 8-days drinking period and for another 3 days (days 9–11). The samples were stored at -20°C until analyzed for ethanol (GC), EtG (LC-MS), 5HTOL (GC-MS) and 5HIAA (HPLC).

Study III

Fresh clinical urine specimens (n = 46) with confirmed bacterial growth were selected to include different common human pathogens. The samples were supplemented with 1 mg/L each of EtG (Medichem Diagnostics, Steinenbronn, Germany) and EtS (TCI, Tokyo, Japan) and were then split into test tubes (without preservatives) that were placed at -20°, 4° and 22°C. Urine samples without the addition of EtG and EtS served as controls. At the start and after 1, 2 and 5 days of storages at 4° and 22°C, samples were placed at -20°C until taken for analysis of EtG and EtS by LC-MS. In a separate experiment, fresh clinical urine specimens (n = 8) confirmed to be positive for *E. coli* were supplemented with 1 mg/L each of EtG and EtS and incubated in test tubes containing sodium fluoride (10 mg NaF/mL urine) as

preservative or without any additive. At the start and after 5 days of storage at 22°C, samples were placed at -20°C until taken for analysis of EtG and EtS by LC-MS.

The studies were approved by the ethics committee at the Karolinska Institutet (Dnr KI 99-338).

Analytical Methods

Ethanol

In *Study I*, ethanol was determined enzymatically using yeast ADH (Helander et al., 1988) on a Hitachi 917 analyzer (La Roche Ltd, Basel, Switzerland). The limit of quantification (LOQ) of this method was 0.5 mmol/L. In *Study II*, ethanol was determined by a headspace GC method with an LOQ of 0.1 mmol/L (Sarkola and Eriksson, 2001).

EtG and EtS

Urinary EtG and EtS were determined by a negative ion electrospray LC-MS method (Stephanson et al., 2002, Helander and Beck, 2004). The LC-MS system used for *Study I* and *II* was a Perkin-Elmer 200 LC and a Sciex API 2000 MS, and in *Study III* an Agilent 1100 series LC-MS. All urine samples, controls and standards were stored at -20°C until use. Before analysis, they were mixed and centrifuged and a 10- μ L aliquot of urine, control or standard was mixed (1:10) with 90 μ L of the internal standard solution (deuterium-labeled EtG and EtS; EtG-d₅ and EtS-d₅) and transferred to autosampler vials. Of the mixture, 10 μ L were injected directly into the LC-MS system, which was equipped with a 5- μ m Hypercarb analytic column (100 x 2.1 mm i.d.). The mobile phase consisted of 25 mmol/L formic acid containing 5% acetonitrile and the flow rate was 0.2 mL/min. Analysis was performed in the negative ion mode, with selected ion monitoring (SIM) of m/z 221 for EtG, m/z 226 for EtG-d₅, m/z 125 for EtS and m/z 130 for EtS-d₅. The concentrations of EtG and EtS were calculated from the peak-area ratios to the internal standards, by reference to the calibration curves. The LOQ of the method was 0.10 mg/L for EtG and EtS, the intra- and interassay coefficients of variation (CV) were less than 12%, and the range of linearity was 0.1–1500 mg/L (Stephanson et al., 2002, Helander and Beck, 2004).

5HTOL

Urinary 5HTOL was determined by GC-MS using an HP 5972 (Hewlett Packard, Palo Alto, CA) (Helander et al., 1996). 5HTOL is mainly excreted in conjugated form with glucuronic acid (Beck et al., 1982; Helander et al., 1995). Before analysis, the urine samples are spiked with an internal standard (deuterium-labeled 5HTOL; 5HTOL-d₄) and incubated at 37°C for 1 h with β-glucuronidase (b-D Glucuronoside glucuronosohydrolase, EC 3.2.1.31 from *E. coli*), resulting in a complete hydrolysis of the 5HTOL glucuronide. The free 5HTOL was then extracted with diethyl ether and finally derivatised. Separation was achieved on a DB1701 capillary GC column (30 m x 0.25 mm i.d.; J&W Scientific, Folsom, CA). GC-MS analysis was performed with selected ion monitoring (SIM) of *m/z* 451 for 5HTOL and *m/z* 454 for 5HTOL-d₄. The concentration of 5HTOL was calculated from the peak-area ratios to the internal standard by reference to a calibration curve. The LOQ of the method was 25 nmol/L.

5HIAA

Urinary 5HIAA was determined by high-performance liquid chromatography (HPLC) with electrochemical detection (Helander et al., 1991). Before analysis, the urine samples were spiked with an internal standard (5-hydroxyindole-2-carboxylic acid). Separation of 5HIAA was achieved on a Nucleosil-120 C₁₈ analytic column (50 x 4.0 mm i.d.). The mobile phase consisted 105 mmol/L citric acid, 12 mL/L methanol, 50 μmol/L EDTA and 25 μmol/L sodium octylsulfate (pH 2.2) and the flow rate was 1 mL/min. The LOQ of the method was 1 μmol/L.

Creatinine

Analysis of creatinine is performed in clinical practice to evaluate kidney function, but also as a way to monitor and compensate for the degree of urine dilution (Lafolie et al., 1991). In *study I*, creatinine was determined by the routine Jaffé reaction, creatinine reacts with picric acid under alkaline conditions to form a red-colored product that is measured on a Hitachi 917 analyzer. The LOQ of the method was 0.5 mmol/L. In *study II*, creatinine was determined by the VITROS CREA Slides which is a multilayered analytical element coated on a polyester support where the final reaction produces a colored product that was measured on a VITROS 250 Chemistry System (Ortho Clinical Diagnostics, Rochester, NY). The LOQ of the method was 0.08 mmol/L.

Pathogens in human urine specimens

Human urine specimens with confirmed growth of different common human pathogens at densities of 10^3 to $>10^5$ colony-forming units (CFU)/mL, as identified and quantified by culture on standard solid media, were obtained from the Department of Clinical Microbiology at the Karolinska University Hospital, Solna, Sweden.

RESULTS AND DISCUSSION

Study I

According to self-report, all participants taking part in this study had abstained from alcohol beverages for at least 48 h. This was confirmed by the observation that all urine samples collected immediately before starting drinking ethanol were negative for ethanol and EtG. Intake of ethanol at 0.5 g/kg (594–986 mL beer; 25.0–41.5 g) resulted in a marked increase in urine volume and a concomitant drop in the urinary creatinine concentration. The ethanol concentration increased rapidly and reached a peak value of 17.0 ± 2.5 mmol/L (mean \pm SD) at 1.5 h after start of the experiment (Figure 3A). The EtG concentration also started to increase and was detectable already in the first urine collection after ethanol intake at 1 h (Figure 3B). At 3 h after starting drinking ethanol, the same volume of tap water was ingested and this produced another increase in urine volume and a drop in the creatinine concentration. The ethanol excretion curve was not influenced by the water intake (Figure 3A), whereas this caused a marked decrease in the rising EtG concentration curve from 44.6 ± 22.6 mg/L at 3 h to 13.8 ± 7.9 mg/L 1 h later (Figure 3B). The EtG concentration increased again after 5.5 h. At 6 h after ethanol intake, ethanol was no longer measurable in urine and the EtG concentration started to fall with a half-life of ~ 2.5 h.

When EtG was expressed relative to the creatinine concentration, this ratio was not markedly influenced by the water intake (Figure 3C). The urinary EtG/creatinine ratio reached a peak value of 8.8 ± 3.5 mg/mmol in the 4-h collection, and thereafter the ratio value decreased with a half-life of ~ 2.5 h, which is in good agreement with previous observations (Schmitt et al., 1997). There were considerable variations in the EtG/creatinine excretion profiles between different individuals.

After the intake of 0.5 g/kg ethanol, ethanol was detectable in urine for 6 h whereas EtG was detectable for 22.5–31.5 h, albeit in the end usually at levels below 1 mg/L. It was

calculated that less than 0.03% (range 0.013–0.025%) of the 0.5 g/kg ethanol dose consumed was recovered in the urine as EtG.

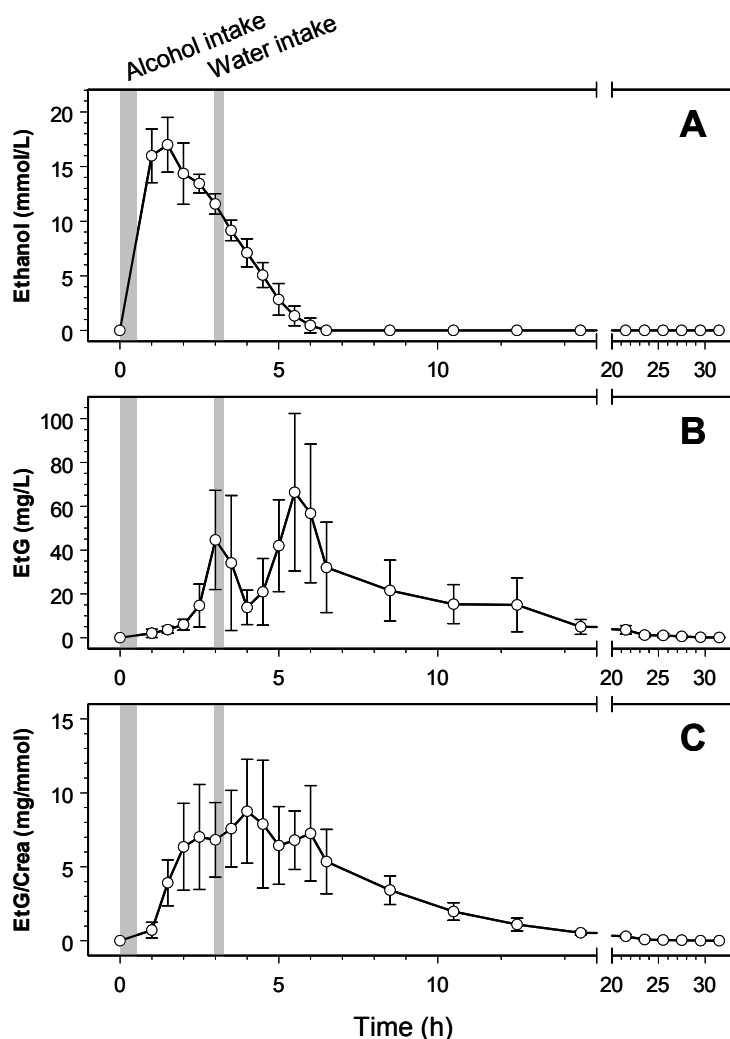


Figure 3. Time-course of diuresis and the urinary concentrations of ethanol (A) and EtG (B), and the urinary EtG/creatinine ratio (C), after six healthy volunteers drank 594–986 mL export beer (corresponding to 0.5 g/kg) in 30 min and 3 h later the same volume of water.

The interest in EtG as a biochemical marker of acute alcohol intake relates to EtG being a direct metabolite of ethanol, and for being detectable several hours longer than ethanol, implying both a high specificity and sensitivity for alcohol (Schmitt et al., 1997; Wurst et al., 1999). The high sensitivity of EtG was confirmed by the observation that intake of a very low dose (~7 g) of ethanol resulted in detectable EtG levels for at least 6 h (Stephanson et al., 2002). Furthermore, following heavy drinking, EtG has been reported to be detectable for up to 4 days after the last ethanol intake (Seidl et al., 2001). EtG can also be detected in samples of hair, body fluids and tissues (Wurst et al., 1999; Pragst et al., 2000; Skopp et al., 2000; Alt et al., 2000; Janda et al., 2002).

The results of the present study showed that, compared with ethanol, EtG was detectable in the urine for an extra ~15–25 h, albeit in the end mostly at low concentrations (<1 mg/L). Of the administered ethanol dose (0.5 g/kg), only about 0.02% was recovered in urine as EtG. This is considerably lower than the 0.5–1.6% previously reported from studies on rabbits (Kamil et al., 1952). Whether the relative formation of EtG in humans is dose-dependent remains to be elucidated.

The present study also pointed at one important limitation of this new alcohol marker. It was demonstrated that ingestion of a water load prior to urine sampling lead to a dramatic reduction in the EtG concentration, whereas, in agreement with previous observations (Bendtsen and Jones, 1999), this did not influence the concentration of ethanol. Accordingly, it is possible to decrease the urinary concentration of EtG to fall below the limit of quantification of the analytic method, simply by drinking moderate to large amounts of water or any other fluid prior to voiding. Internal and external dilution of the urine are well-known strategies among drug abusers, to try to avoid detection when being tested for illicit drug use (Wu, 1998).

This study also demonstrated that the interference by water-induced diuresis on the EtG concentration could be overcome, by expressing EtG as a ratio to creatinine. Normalization of values to creatinine is common practice to compensate for unusually dilute or concentrated urine samples (Lafolie et al., 1991; Needleman et al., 1992; Bendtsen and Jones, 1999). However, even when EtG was expressed as a ratio to creatinine, there were considerable inter-individual variations in the excretions profiles, which was later confirmed by others (Goll et al., 2002). The individual variation could depend on factors such as differences in ethanol distribution and elimination, enzyme activities, enzyme induction, and polymorphism of the UGT enzymes (de Wildt et al., 1999).

In summary, the results of the present study confirmed that EtG remains detectable in the urine for several hours after the ethanol itself has been eliminated. The results also showed that it is possible to markedly lower the urinary concentration of EtG simply by drinking large amount of water or fluid prior to voiding, whereas this did not influence the concentration of ethanol or the EtG/creatinine ratio. Checking the urine for an abnormally low creatinine concentration or expressing urinary EtG as a ratio to creatinine, therefore may be recommended in routine clinical use, to detect and compensate for urine dilution. It was also demonstrated that conjugation of ethanol with glucuronic acid to form EtG represents a very minor elimination pathway for ethanol in humans.

Study II

In this study, healthy female subjects drank ethanol (8%, w/v) in juice at 0.4 g/kg or placebo (juice) twice daily (morning and evening) in random order for 8 consecutive days. Thereafter, they were followed for another 3 days (days 9–11) without ethanol intake. Spot urine samples were collected according to the study protocol. During the placebo drinking period on days 1–8 and also on days 9–11, all urine samples were found to be negative for ethanol and showed 5HTOL/5HIAA ratios below the reference value (<15 nmol/ μ mol). All urine samples except two (two subjects showed one positive EtG value each; 0.60 and 0.12 mg/L) were also negative for EtG.

During the ethanol drinking period on days 3–8, ~56% of the urine samples were found to be positive for ethanol, ~78% showed an increased 5HTOL/5HIAA ratio, and all (100%) were positive for EtG. However, the individual values during the ethanol drinking period were highly variable between subjects (Figure 4). Accordingly, the urinary ethanol values ranged between 0–7.3 mmol/L, the 5HTOL/5HIAA ratio between 2–109 nmol/ μ mol, the EtG/creatinine ratio between 0.1–4.5 mg/mmol, and the EtG concentration between 1.4–71 mg/L. For some individuals, there was also a considerable variation in the results on different days. The urine samples collected on day 9, which was ~14–15 h after the last intake of 0.4 g/kg ethanol, were negative for ethanol and showed a normal 5HTOL/5HIAA ratio, while EtG and the EtG/creatinine ratio were positive. One subject was found positive for EtG (0.26 mg/L) also in the morning on day 10, which was ~38 h after the last intake. This subject showed the highest EtG value (80.5 mg/L) at 4 h after the last intake on day 8.

Compared with the starting values, the ethanol elimination rate was significantly increased by 24% on average at the end of drinking period (day 8) (Sarkola et al., 2001). However, there were no significant differences between the ratios of EtG/creatinine and 5HTOL/5HIAA at 4 h after ethanol intake on days 1 and 8, whereas the EtG/creatinine ratio at 15 h was significantly lower on day 8.

An increased urinary ratio of 5HTOL/5HIAA has been used clinically for several years as a sensitive biochemical marker for recent alcohol consumption (Voltaire et al., 1992; Voltaire Carlsson et al., 1993; Helander et al., 1994; Borg et al., 1995). More recent studies have demonstrated that analysis of EtG in urine can also be useful for this purpose (Schmitt et al., 1997; Wurst et al., 1999). The present results confirmed previous observations that EtG and the urinary 5HTOL/5HIAA ratio both become increased shortly after consumption of alcohol and they remained increased for many hours longer than ethanol itself. In this respect,

the detection time for EtG was found to be longer than for 5HTOL/5HIAA. It is possible that the longer detection time for EtG compared with 5HTOL/5HIAA simply results from the higher concentration of EtG, because the EtG level is more than 100-fold higher than the 5HTOL level after ethanol intake (Helander et al., 1993; Schmitt et al., 1997). As for the urinary 5HTOL/5HIAA ratio, EtG can be expressed as a ratio to creatinine to compensate for variations in the concentration caused by urine dilution (Helander et al., 1992; Dahl et al., 2002).

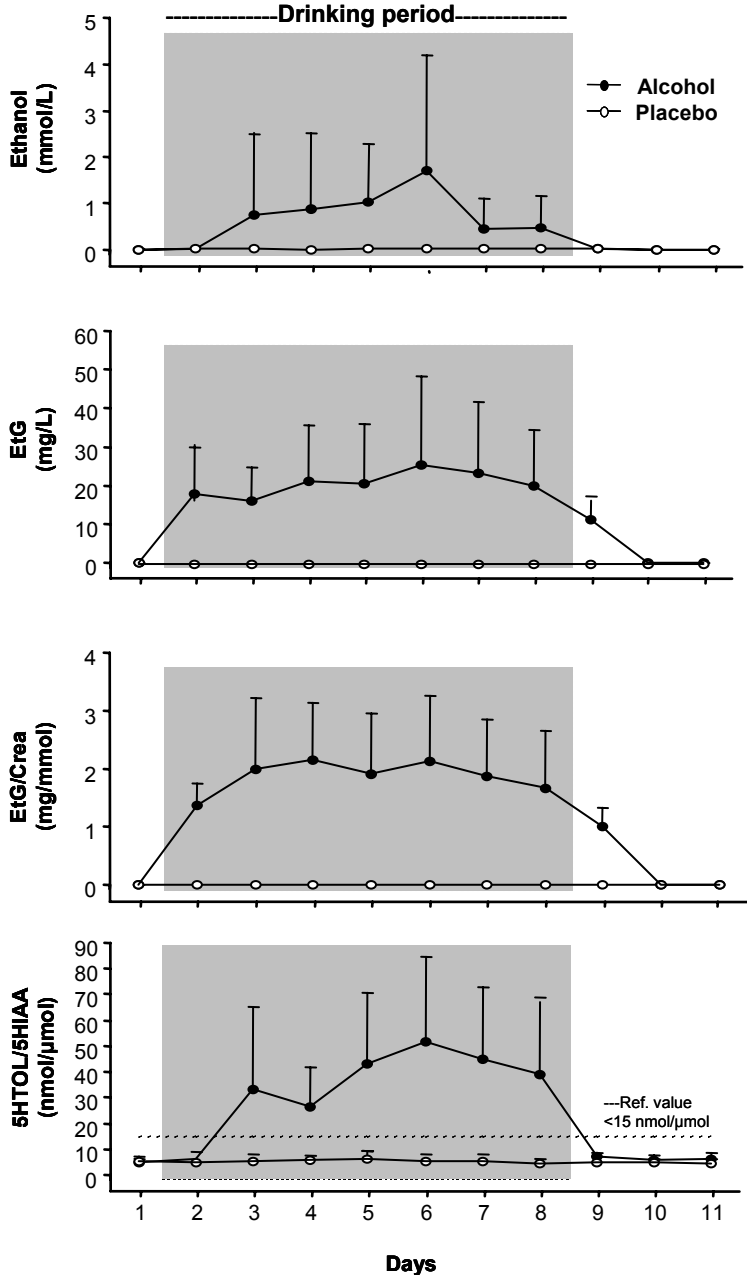


Figure 4. Urinary concentrations of ethanol and EtG, and the ratios of EtG/creatinine and 5HTOL/5HIAA, in first morning urine void samples collected during ethanol (0.4 g/kg twice daily, once in the morning and once in the evening) and placebo drinking periods.

During the placebo period, two subjects showed positive results for urinary EtG, albeit at low levels. It cannot be excluded that these resulted from unintentional intake, as ethanol is also present in so-called “alcohol-free” beverages (<0.5% ethanol). The endogenous ethanol concentration has been estimated not to be high enough to produce a detectable concentration of EtG (Janda and Alt, 2001; Stephanson et al., 2002).

In cases of prolonged heavy drinking, EtG has been reported to be detectable for up to 4 days after the last alcohol intake (Seidl et al., 2001). This observation, together with other reports, suggested that EtG may accumulate in the body upon prolonged drinking (Schmitt et al., 1997; Seidl et al., 2001). However, the present study found no indication of an accumulation of EtG, or a gradually increased 5HTOL/5HIAA ratio, upon repeated ethanol exposure at 0.8 g/kg ethanol per day for a period of ~1 week. After 8 days of drinking, the elimination rate of ethanol was found to be increased by 24% (Sarkola et al., 2001). This observation could possibly explain the significantly lower EtG/creatinine ratio observed at 15 h after the last intake compared with on day 1. Accordingly, the lower EtG/creatinine ratio was most likely related to the lower concentration of ethanol.

During the alcohol drinking period, there were considerable variations in ethanol, EtG, and 5HTOL/5HIAA levels in the morning urine samples, both between and within subjects. This was probably due to a combination of effects such as occasional voiding during nighttime, and some hours difference in the time between drinking the ethanol and collecting the urine sample, as well as biological variations. The variability for EtG could not be explained simply by urine dilution, as it was evident also when EtG was normalized to creatinine.

In summary, the results of this study confirmed that the urinary excretion of EtG and 5HTOL become increased following acute ethanol intake, and also remains detectable for many hours after the ethanol is no longer measurable. There were no significant accumulation in the body of either EtG or 5HTOL, upon multiple-dose administration of ethanol at 0.8 g/kg per day for ~1 week. This study therefore supports the use of urinary EtG and an elevated 5HTOL/5HIAA ratio as sensitive and specific tests to assess recent drinking in light to moderate as well as chronic alcohol consumers. When used for this purpose, the detection time for EtG was found to be longer than for 5HTOL/5HIAA.

Study III

In the majority (68%) of the urine specimens with confirmed growth of *E. coli*, the EtG concentration was found to decrease over time on storage at 22°C but not at 4° or -20°C

(examples are given in Figure 5). In 3 specimens, a complete hydrolysis of the EtG added (1 mg/L) was noted after 24 h at 22°C. In 2 specimens that contained very high EtG concentrations (37.3 and 39.3 mg/L) already before EtG supplementation, the levels decreased to 11.2 and 0.7 mg/L, respectively, after storage for 5 days at 22°C.

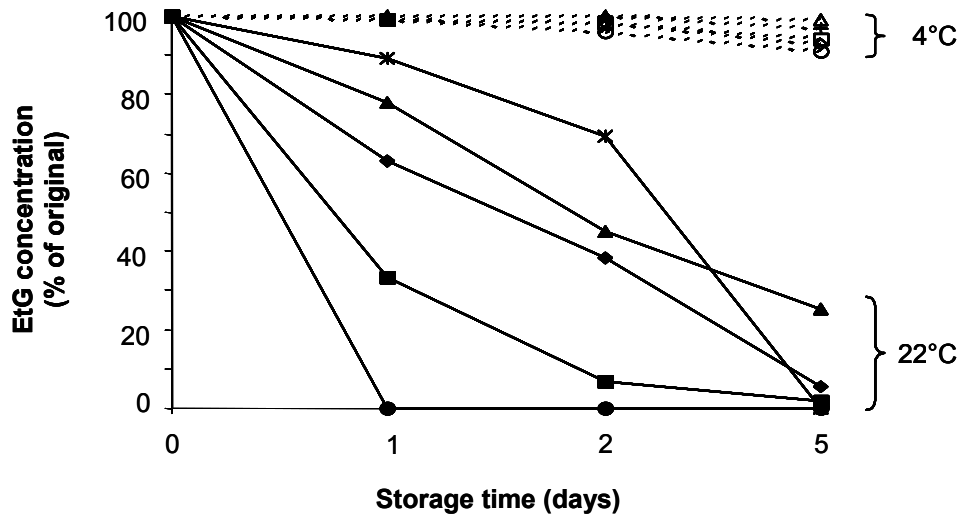


Figure 5. Effect of storage time and temperature on the hydrolysis of EtG by bacterial β -glucuronidase in clinical urine specimens containing *E. coli*.

In 1 of 3 urine specimens containing *Klebsiella pneumoniae* and the single specimen containing *Enterobacter cloacae*, the EtG concentration also decreased over time after storage at 22°C. None of the uropathogens tested (Table 1) caused hydrolysis of EtS after samples were supplemented with 1 mg/L EtS and stored at 22°C for 1-5 days.

To evaluate the effect of chemical preservatives, 8 urine specimens confirmed positive for *E. coli* were supplemented with 1 mg/L each of EtG and EtS and the volume was then split into two test tubes, one containing preservative (10 mg NaF/mL urine) and the other without any additive. The samples were then stored at 22°C for 5 days. In 6 specimens, hydrolysis of EtG was observed in the tubes without preservatives after storage at 22°C for 5 days, whereas EtG was found to be completely stable on storage in the tubes containing NaF. No decrease in the EtS concentration on storage was observed for any of these samples.

After consumption of alcohol beverages, a very small fraction (<0.1%) of the ingested ethanol undergoes conjugation reactions to produce EtG and EtS (Dahl et al., 2002; Helander and Beck, 2004). EtG and more recently EtS have thus been introduced as sensitive and specific biochemical markers of recent alcohol intake (Seidl et al., 2001; Skipper et al., 2004;

Schneider and Glatt, 2004; Dresen et al., 2004; Helander and Beck, 2005). However, glucuronide and sulfate conjugates are hydrolyzed by β -glucuronidase and sulfatase enzymes. β -Glucuronidase is present with high activity in most strains of *E. coli*, which is the most common bacterium isolated in clinical laboratories and also the predominant pathogen (~80%) in urinary tract infections (Ronald, 2002). Sulfatase activity has also been detected in many bacteria (Kertesz, 2001) but not in *E. coli* (Murooka et al., 1978; Dealler et al., 1992), or only in very low amounts (O'Brien and Herschlag, 1998).

Table 1. Uropathogens tested for causing falsely low or false-negative EtG or EtS results due to 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>Escherichia coli</i>	25	17 (68)	0
<i>Enterococcus species</i>	5	0 (0)	0
Group B streptococci	3	0 (0)	0
<i>Klebsiella pneumoniae</i>	3	1 (33)	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>Enterobacter cloacae</i>	1	1 (100)	0
<i>Enterobacter aerogenes</i>	1	0 (0)	0
<i>Morganella morganii</i>	1	0 (0)	0

^a EtG and EtS (1 mg/L 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.

The present study demonstrated that bacterial β -glucuronidase, especially if samples are positive for *E. coli*, can hydrolyze EtG and cause falsely low or false-negative results. These results agree with previous studies showing that most *E. coli* strains possess β -glucuronidase activity (Hansen and Yourassowsky, 1984; Tapsall and McIver, 1995; Dalet and Segovia, 1995). *Klebsiella pneumoniae* and *Enterobacter cloacae* also caused a gradual disappearance of EtG, but the rate of EtG hydrolysis was typically slower than for *E. coli* under the same conditions. These observations are consistent with previous results, which indicate that other pathogens also possess low β -glucuronidase activity (Tapsall and McIver, 1995; Dalet and Segovia, 1995). Our results therefore concluded that EtG might not be stable on storage, if urine specimens taken for analysis are infected with pathogens possessing β -glucuronidase

activity. In contrast to EtG, EtS was indicated to be completely stable to bacterial hydrolysis. The disappearance of EtG was found to be temperature dependent, with refrigeration or freezing of samples, or use of test tubes containing NaF as preservative, being effective to prevent hydrolysis.

In summary, the present study demonstrated that EtG but not EtS is sensitive to bacterial hydrolysis, particularly in specimens infected by *E. coli*. To prevent bacterial growth and hydrolysis of EtG, the urine specimens should be refrigerated or frozen. Using test tubes containing NaF is also recommended. Since EtS shows a similar time course as EtG after alcohol consumption, it may be recommended, and an analytical advantage, to measure EtS instead of EtG, or to combine EtG with EtS which is possible by LC-MS (Helander and Beck, 2004; Helander and Beck, 2005).

CONCLUSIONS

The present results demonstrated that conjugation of ethanol to produce EtG represents a very minor elimination pathway (<0.03%) for ethanol in humans. The results further confirmed previous observations that EtG remains detectable in the urine for many hours after ethanol itself has been eliminated from the body and is no longer measurable. The detection time for urinary EtG was found to be longer than for the 5HTOL/5HIAA ratio. There was no significant accumulation of either EtG or 5HTOL upon multiple-dose administration of ethanol, indicating that both markers are useful to detect recent drinking in light as well as heavy alcohol consumers. By drinking large volumes of water or fluid prior to voiding, it is possible to lower the urinary concentration of EtG, whereas this did not influence the concentration of ethanol or the EtG/creatinine ratio. To compensate for urine dilution, it is therefore recommended to express urinary EtG as a ratio to creatinine or to check samples for an abnormally low creatinine content. The present results further demonstrated that EtG but not EtS is sensitive to bacterial hydrolysis, particularly in specimens infected by *E. coli*. To prevent bacterial growth and hydrolysis of EtG, the specimens should be stored refrigerated or frozen, or samples should be collected in test tubes containing NaF as preservative. In clinical practice, it may be recommended to combine EtG and EtS testing, which is possible by LC-MS. Accordingly, as previously shown for 5HTOL/5HIAA, urinary EtG and EtS can be used as sensitive and specific biochemical markers for acute alcohol intake.

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