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Evaluation and clinical application of ethyl glucuronide and ethyl sulfate as biomarkers for recent alcohol consumption

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**Karolinska
Institutet**

Stockholm 2011

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ISBN 978-91-7457-221-6

Printed by



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Gårdsvägen 4, 169 70 Solna



♥ *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: "short-term markers" and "long-term markers". Short-term markers are sensitive enough to detect a single intake of alcohol e.g. ethanol, 5-hydroxytryptophol (5HTOL), ethyl glucuronide (EtG) and ethyl sulfate (EtS). Long-term markers detect chronic heavy drinking, or indicate body organ or tissue damage caused by long-term exposure to alcohol e.g. carbohydrate-deficient transferrin (CDT), phosphatidylethanol (PEth), γ -glutamyl transferase (GGT), aspartate and alanine aminotransferase (AST and ALT), and the mean corpuscular volume of erythrocytes (MCV).

Following alcohol consumption less than 5% of the ethanol is excreted unchanged via the urine, sweat and breath, while more than 95% instead becomes metabolized mainly in the liver in a two-stage oxidation process. A minor 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, thereby allowing a longer detection time. A positive finding of EtG and/or EtS in urine or plasma thus provides a strong indication that the person recently drank alcohol, even if drinking is denied, since levels of EtG and EtS remain elevated for some time after ethanol itself is no longer detectable.

The purpose of this thesis was to evaluate the accuracy of urinary EtG and EtS measurement and the clinical application as biochemical markers for acute alcohol consumption. Urinary EtG and EtS were determined by liquid chromatography-mass spectrometry (LC-MS).

The current studies demonstrated that EtG is a direct metabolite of ethanol and represents a minor elimination pathway (<0.03%) in the human body and 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 to lower the urinary concentration of EtG, but this practice did not influence the concentration of the EtG/creatinine ratio, no significant accumulation of EtG or 5HTOL was observed upon multiple-dose administration of ethanol, and the detection time in urine for EtG was demonstrated to be longer than for 5HTOL. It was found that EtG but not EtS is sensitive to bacterial hydrolysis. To reduce the risk for obtaining false low or false-negative EtG results specimens should be stored refrigerated or frozen prior to analysis. Plasma EtG was found useful in the emergency department to detect recent drinking even when ethanol is negative to confirm alcohol abstinence. In 87% of the cases the information about recent drinking provided by self-report agreed with the EtG and EtS results in an outpatient treatment program for alcohol and drug dependence. EtG and EtS may also be objective outcome measures when evaluating new treatment strategies and pharmacotherapies.

In conclusion, the present results demonstrated that urinary EtG and EtS are very sensitive and specific biochemical markers for acute alcohol intake.

LIST OF PUBLICATIONS

- 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.
- IV. Neumann T, Helander A, Dahl H, Holzmann T, Neuner B, Weiss-Gerlach E, Muller C and Spies C. (2008). Value of ethyl glucuronide in plasma as a biomarker for recent alcohol consumption in the emergency room. *Alcohol and Alcoholism*. 43, 431-5.
- V. Dahl H, Voltaire Carlsson A, Hillgren K, Helander A. (2011). Urinary ethyl glucuronide and ethyl sulfate testing for detection of recent drinking in an outpatient treatment program for alcohol and drug dependence. *Alcohol and Alcoholism* (accepted).
- VI. Dahl H, Hammarberg A, Franck J, Helander A. (2011). Urinary ethyl glucuronide and ethyl sulfate testing for recent drinking in alcohol-dependent outpatients treated with acamprosate or placebo. (manuscript).

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LIST OF 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
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
<i>E. coli</i>	<i>Escherichia coli</i>
ED	Emergency department
EtOH	Ethanol
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
ICD-10	International Classification of Diseases, 10 th revision
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
PEth	Phosphatidylethanol
TLFB	Timeline follow-back
UDPGA	Uridine-5-diphospho- β -glucuronic acid
UGT	UDP-glucuronosyltransferase
UTI	Urinary tract infection

1. INTRODUCTION

Alcohol is one of the most widely used addictive substances in the world and has been a part of human culture since the beginning of recorded history (1). Almost all societies that consume alcohol show related health and social problems. 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 (2). The use and abuse of alcohol cost the Swedish society enormous amounts each year, not only related to increased health care costs but to other factors such as lost productivity (2-4).

In a Swedish study carried out in a surgical emergency ward, about 15% of the patients screened positive for hazardous alcohol consumption (5, 6). 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 (7). A Swedish workplace study indicated that individuals with moderately elevated or harmful levels of alcohol consumption show an increase in sick-days (8). 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's 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 (9), CAGE (10) and Mm-MAST (11). However, it is well known that people may not always report their alcohol intake correctly, but often deny drinking or under-report the true amount (12, 13). 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.

2. 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%) (14). The absorption becomes delayed if there is food in the stomach (15). 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 body weight and body fat.

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 (15) (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). 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 the ethanol becomes oxidized to acetaldehyde via CYP2E1, belonging to the cytochrome P450 family, in the microsomal ethanol oxidizing system (MEOS) or by catalase, 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).

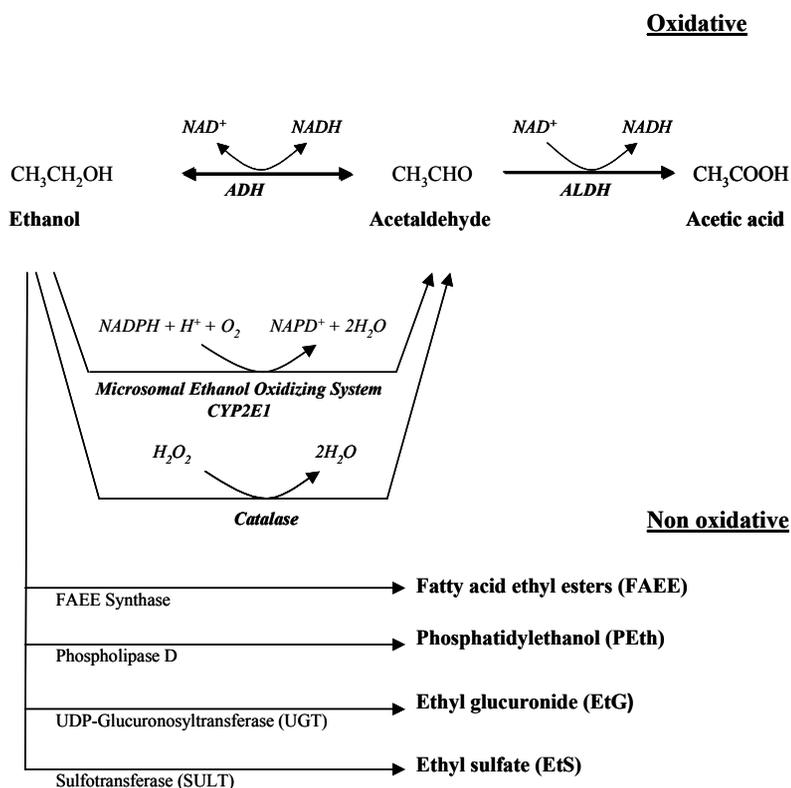


Figure 1. The major oxidative pathway and minor non-oxidative pathways for ethanol elimination in the human body.

3. ALCOHOL DEPENDENCE

The AUDIT was developed by the WHO as a simple screening test to identify persons with hazardous, harmful or dependence patterns of alcohol consumption (16). Hazardous drinking is a pattern of alcohol consumption that increases the risk of harmful consequences for the user and results in consequences to physical and mental health. Alcohol-dependent persons show a behavior of strong desire to consume alcohol, impaired control over the use, persistent drinking despite harmful consequences, higher priority given to drinking than other activities and obligations, and an increased alcohol tolerance and physical withdrawal reaction when using alcohol (17).

The criteria of alcohol dependence has been defined in different classification systems such as the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) published by the American Psychiatric Association in 2004 (Table 1), or the International Classification of Diseases, 10th revision (ICD-10) published by the World Health Organisation in 1993.

Table 1. Definition of alcohol abuse according to DSM-IV criteria.

<p>A maladaptive pattern of alcohol use, leading to clinically significant impairment or distress, as manifest by three (or more) of the following, occurring at any time in the same 12-month period.</p> <ol style="list-style-type: none">1. Tolerance, as defined by either of the following:<ol style="list-style-type: none">a) A need for markedly increased amounts of alcohol to achieve intoxication or desired effect.b) Markedly diminished effect with continued use of the same amount of alcohol.2. Withdrawal, as manifested by either of the following:<ol style="list-style-type: none">a) The characteristic withdrawal syndrome for alcohol.b) Alcohol (or a closely related substance) is taken to relieve or avoid withdrawal symptoms.3. Alcohol is often taken in larger amounts or over a longer period than was intended.4. There is a persistent desire or unsuccessful effort to cut down or control alcohol use.5. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from effects.6. Important social, occupational, or recreational activities are given up or reduced because of alcohol.7. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
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4. PHARMACOLOGICAL MEDICATIONS

Treatment of alcohol dependence usually combines with psychosocial interventions, such as motivational interviewing (18, 19), cognitive behavioral therapy (20), 12-step treatment from the Alcoholic Anonymous model and pharmacological medications.

Disulfiram (Antabuse[®]) is a well known drug which has been used clinically since the late 1940s. The mechanism of action is by inhibiting the degradation of alcohol by inhibition of ALDH. This causes an accumulation of acetaldehyde, the first metabolite of ethanol oxidation (Figure 1). Increasing levels of acetaldehyde leads to unpleasant effects such as flushing, tachycardia, headache and nausea.

Naltrexone (Revia[®]) is a non-selective opioid antagonist, which has shown to reduce the rate of relapse into heavy drinking as well the as craving for alcohol (21).

Acamprosate (calcium acetyl homotaurinate; Campral[®]), a modulator of glutamate neurotransmission, has demonstrated some ability to reduce drinking and increase the time spent abstinent (22-24).

5. 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 (25-28). 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 (29), workplace settings (30), traffic medicine (31), and forensic toxicology (32). The biochemical alcohol markers are often distinguished into the following main classes:

1. Markers of acute alcohol consumption (short-term markers) 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) (33, 34), EtG (35, 36) and EtS (37).

2. Markers of chronic, heavy alcohol consumption (long-term markers) are laboratory test that can detect sustained, harmful intake of alcohol, and include carbohydrate-deficient transferrin (CDT) (38), PEth (39, 40), and the mean corpuscular volume of erythrocytes (MCV) (41), 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) (41) and aspartate and alanine aminotransferase (AST and ALT) (42).
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 (43) and adenylyl cyclase (44) have, for example, been proposed as trait markers for alcohol dependence.

5.1. 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)

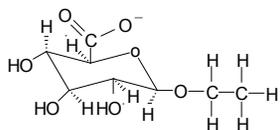
6. MARKERS OF ACUTE ALCOHOL CONSUMPTION

6.1. Ethyl glucuronide (EtG)

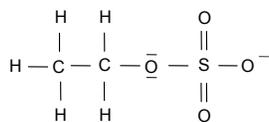
EtG is a minor non-oxidative direct metabolite of ethanol that is formed by reaction with uridine-5-diphospho- β -glucuronic acid (UDPGA) catalyzed by the endoplasmic reticulum UDP-glucuronosyltransferase (UGT) enzymes (45-47). The pathway of ethanol elimination via conjugation with UDPGA was first described in 1901. 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 (48). EtG was later determined also in human urine (49-51). In 1995, EtG was synthesized and identified in human serum (50). The molecular weight of EtG is 222 g/mol and of penta-deuterated EtG (EtG-d₅) used as internal standard 227 g/mol (Figure 2).

The renewed interest in EtG is primarily related to its use as a biochemical marker for acute alcohol intake. This interest originates from the observation that the washout rate for EtG is much slower than for ethanol (35, 36), but also from the development of sensitive and specific analytical methods based on gas chromatographic-mass spectrometric (GC-MS) (36) and liquid chromatographic-mass spectrometric (LC-MS) techniques (52). 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 (35) and to show high inter-individual variation with a poor correlation with the concentration of ethanol (53). The latter observation can at least partly be explained by the time-lag between the excretion profiles for ethanol and EtG.

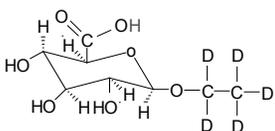
The high sensitivity of EtG for recent drinking is evident from the observation that even intake of a very low dose (~7 g) of ethanol is detectable in the urine after 6 h (52). Depending on the amount of ethanol consumed, EtG has been reported to be measurable in the urine for up to 4 days after the last intake (54) (Figure 3). Except for urine and blood, EtG can also be detected in samples of hair (55-58), oral fluid (59), and in tissue extracts (60, 61).



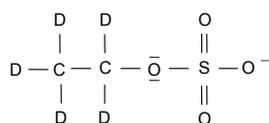
EtG (M_w 222)



EtS (M_w 126)



EtG-d₅ (M_w 227)



EtS-d₅ (M_w 131)

Figure 2. The molecular structures of EtG, EtG-d₅, EtS and EtS-d₅.

6.2. Ethyl sulfate (EtS)

EtS is another conjugated, direct ethanol metabolite that is formed by reaction with sulfate by the action of cytosolic sulfotransferase (62-65). In humans, the elimination pathway for ethanol via sulfate conjugation to produce EtS was first established in 2004 by LC-MS (66). The molecular weight of EtS is 126 g/mol and of penta-deuterated EtS (EtS-d₅) 131 g/mol (Figure 2). The human study also demonstrated that only a very small fraction (<0.1%) of the ingested ethanol undergoes sulfate conjugation. Furthermore, EtS showed very similar urinary excretion profiles as EtG, but EtS was typically present in lower amounts compared with EtG. Depending on the amount of ethanol consumed, EtS has been reported to be measurable in the urine for up to 4 days after the last intake (54) (Figure 3). Recently there has been an increased focus on EtS as a biochemical marker for acute alcohol intake (37, 67-70).

6.3. Fatty acid ethyl esters (FAEE)

FAEE are products of non-oxidative metabolism of ethanol via FAEE synthase (71). FAEE have been proposed as a sensitive and specific biochemical marker of acute alcohol intake (72, 73). FAEE can be detected for up to at least 24 h after alcohol intake (74), although a recent study reported a faster elimination of FAEE compared with earlier studies (75) (Figure 3). FAEE may also be important in the pathogenesis of organ damage caused by alcohol abuse, since high FAEE concentrations are found in alcohol-related organ damage (76).

6.4. 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 (77) (Figure 3). 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 (78). This metabolic shift from the oxidative towards the reductive 5HT pathway occurs because of competitive inhibition of mitochondrial ALDH by acetaldehyde and the increased cytosolic NADH/NAD⁺ ratio, both favouring 5HTOL formation at the expense of 5HIAA (79, 80). Furthermore, the reoxidation of 5HTOL to 5HIAL by ADH is inhibited during ethanol oxidation (81).

Soon after drinking alcohol, the urinary 5HTOL concentration becomes markedly increased and will not return to baseline levels until several hours after the ethanol is no longer measurable (78) (Figure 3). Based on this time-lag, urinary 5HTOL has been used clinically as a sensitive biochemical marker of recent alcohol consumption (33, 82). 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 (83). Gender or genetic variations in the ADH and ALDH isoenzyme patterns seemingly do not influence the baseline ratio of 5HTOL/5HIAA (34, 84). 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[®]) (85). 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 (86, 87). 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) (83, 88).

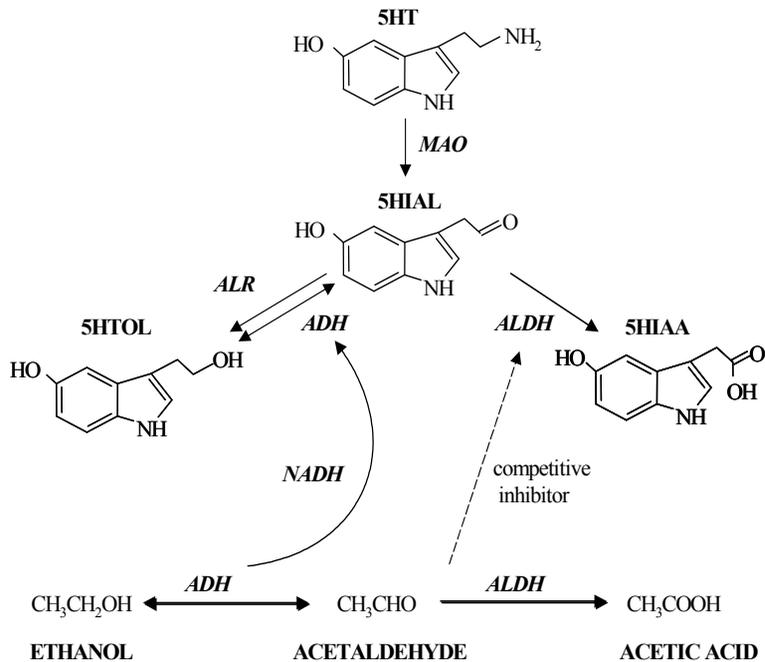


Figure 3. The interaction between serotonin (5HT) and ethanol metabolism.

7. MARKERS OF CHRONIC ALCOHOL CONSUMPTION

7.1. Carbohydrate-deficient transferrin (CDT)

Transferrin is a glycoprotein that is synthesized by the liver with the specific function to transport iron (Fe^{3+}) in the body (89). There is a glycoform microheterogeneity of human transferrin, usually resulting in non-detectable levels of asialo- and monosialo-, <2% disialo-, 4.5–9% trisialo-, 64–80% tetrasialo-, 12–18% pentasialo-, and 1–3% hexasialotransferrin in serum (90-92). The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has identified disialotransferrin as the main target analyte for the alcohol marker CDT (93). After prolonged heavy alcohol consumption the glycoform pattern changes towards a higher concentration of disialo- and asialotransferrin (38, 94-98). The rather alcohol-specific increase of CDT makes it useful as a marker for chronic alcohol consumption (99, 100). To increase CDT, a relative large daily alcohol consumption is required, corresponding to ~50–80 gram ethanol per day for ~2 weeks or longer (38, 101-103) (Figure 4). CDT has a half-life of 1.5–2 weeks (38, 95, 99, 104). Methods to analyze CDT are for example a candidate reference HPLC method (105) and commercial assays based on immunochemistry, capillary zone electrophoresis or HPLC.

7.2. Phosphatidylethanol (PEth)

PEth is a group of abnormal phospholipids which are formed only in the presence of ethanol via the action of phospholipase D (39, 40, 106). Normally phospholipase D uses water as substrate to produce phosphatidic acid. PEth has a half-life of ~4 days and is detectable in alcohol abusers up to 2–4 weeks after alcohol withdrawal (107, 108) (Figure 4). The PEth concentration in blood correlates with the amount of ethanol intake in alcohol abusers (108, 109) and it is thus a promising new biomarker of chronic alcohol use (110-112). The current methods used for PEth analysis include HPLC (108, 113) and LC-MS (40, 114).

7.3. Gamma-glutamyltransferase (GGT)

The enzyme GGT is a non-specific index of liver damage and is also used as a biomarker for chronic alcohol consumption (96, 115, 116). The half-life of GGT is 2–3 weeks and the level usually returns to normal within 4-5 weeks after drinking is discontinued (96, 117-119) (Figure 4). The drinking intensity and frequency and even regular intake of low alcohol levels (1–40 g/day) may affect GGT (115, 120). There are many other causes for an elevated GGT such as drugs, diabetes, smoking, age, gender and non-alcoholic liver disease (121-124).

7.4. Aspartate and alanine aminotransferase (AST and ALT)

The enzymes AST and ALT are non-specific indicators of liver and tissue damage that are also used as biomarkers for chronic alcohol consumption (25, 125-128) (Figure 4). The half-life of AST and ALT is 2–3 weeks (118). The sensitivity and specificity of AST and ALT for alcohol are fairly low (118, 129). Calculation of the AST/ALT ratio may help to differentiate between alcoholic and non-alcoholic liver damage, with an enzyme ratio over 2.0 suggestive of alcohol aetiology (115, 123, 128). There are also many other causes for an elevated AST such as muscle disorders, myocardial infarction, skeletal muscle trauma and non-alcoholic liver disease (42, 118).

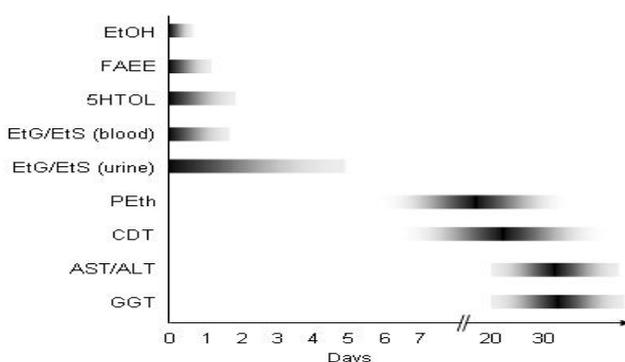


Figure 4. Estimated detection times for alcohol intake by different markers.

8. AIMS

8.1. General aim

The purpose of this work was to evaluate the accuracy of urinary EtG and EtS as biochemical markers 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 and EtS results.

8.2. 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 may cause hydrolysis of EtG and/or EtS by bacterial β -glucuronidase and sulfatase enzymes, hence resulting in false-negative results, and by that to investigate the impact of urine sample handling.
- To determine the value of plasma EtG as a marker to obtain information about alcohol consumption in an emergency department.
- To determine the information about recent alcohol consumption obtained by urinary EtG and EtS when introduced as a routine test in an outpatient treatment program for alcohol and drug dependence.
- To examine the value of urinary EtG and EtS testing compared with self-reports for detection of prior drinking in alcohol-dependent outpatients.

9. MATERIALS AND METHODS

9.1. 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 of EtG by LC-MS (52).

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 the 3 following days (days 9–11). The samples were stored at -20°C until analyzed of ethanol by GC, EtG by LC-MS, 5HTOL by GC-MS and 5HIAA by 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 and EtS 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 storage 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.

Study IV

Male patients (n = 81) aged 18–76 years admitted to the emergency department (ED) and diagnosed to be minimally injured and clinically non-intoxicated participated in this study. A detailed description of the experimental design is found elsewhere (130). The patients were asked to complete a computerized alcohol questionnaire including the 10 questions of the standardized AUDIT questionnaire (16, 131) and a self-report (paper and pencil) questionnaire (130) about alcohol consumption data. The questions included the type and amount of alcoholic beverage consumed on weekdays and during weekends, and the time, type and amount of last intake. To adjust for the variable doses of alcohol and different times between drinking and blood sampling the estimated time since completed ethanol elimination was calculated for each patient by using an elimination rate of 0.15 g/kg/h and a distribution volume of $0.7 \times$ body weight. Blood was collected and stored at -20°C until analyzed for EtG by LC-MS.

Study V

A total of 24 patients (21 men and 3 women, aged 21–52 years) with alcohol and/or drug dependency undergoing outpatient treatment agreed to participate in this study. In connection with regular return visit to the clinic patients were invited to leave a urine sample and complete a single-question form about recent drinking (“Did you drink any alcohol in the past 3 days – Yes/No?”). The urine sample and the single-question form were de-identified and only connected by a code with no possibility to track the patient. The subjects were allocated to 3 sub-groups depending on the type of outpatient treatment program: Group *A* included 8 individuals (7 men and 1 woman) undergoing treatment for alcohol dependence; Group *D* included 10 individuals (9 men and 1 woman) being treated for drug dependence (former heroin addicts); and Group *M* included 6 individuals (5 men and 1 woman) undergoing methadone maintenance therapy for opioid addiction. The samples were placed at -20°C until taken for analysis of EtG and EtS by LC-MS.

Study VI

This study involved 56 treatment-seeking individuals (30 men and 26 women, mean age 50 years) recruited via advertisements in a local newspaper. The inclusion criteria were a male or a non-pregnant/non-nursing female age 18–65 years fulfilling criteria for alcohol dependence according to DSM-IV and with a goal of controlled drinking, willingness to give informed consent, complying with the procedures, and having consumed alcohol on at least 15 of the past 90 days according to self-report. Exclusion criteria were seeking complete abstinence, a psychiatric disorder diagnosis according to DSM-IV (including all forms of substance dependence other than nicotine and alcohol), current use of psychoactive medications to manage schizophrenia, a bipolar disorder or major depression, inpatient alcohol detoxification within the last 4 days, acamprostate medication during the last 12 months, and use of illegal drugs during the course of the study. The patients were randomized to 21 days of either oral acamprostate (2 g/day) or placebo treatment in a double-blind design, as described in detail elsewhere (132). Return visits to the ward for blood and urine sampling and filling out alcohol questionnaires were made on day 7, 14 (leaving urine was optional on these days) and 22 (a urine sample was mandatory). On day 22, they were also required to provide a negative breath alcohol test. The questionnaire focused on prior alcohol consumption, expressed as the number of standard drinks (1 drink = 12 g ethanol) per day over the past 3 days, using a time-line follow-back method. The patients were instructed to refrain from alcohol during the treatment period, evidence of drinking was not grounds for study discontinuation unless warranted for clinical safety. The samples were placed at -20°C until taken for analysis of EtG and EtS by LC-MS.

9.2. Ethical approval

Study I–III and *V* was approved by the ethics committee at the Karolinska Institutet (Dnr KI 99-338). *Study IV* obtained ethical approval from the International Review Board of the University Hospital Charité, Berlin, Germany (EK-Vorg: 1514/2001). *Study VI* was approved by the ethics committee at the Karolinska Institutet (Dnr KI 2007/995-31).

9.3. Analytical methods

Ethanol

In *Study I*, ethanol was determined enzymatically using yeast ADH (133) 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 (134).

EtG and EtS in urine

Urinary EtG and EtS were determined by a negative ion electrospray LC-MS method (52, 66). 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-VI* an Agilent 1100 series LC-MS. All urine samples, controls and standards were stored at -20°C until use. Before analysis, the samples were mixed and centrifuged and a 10-µL aliquot of urine, control or standard was mixed (1:10) with 90 µL of the internal standard solution (deuterium-labelled EtG and EtS; EtG-d₅ and EtS-d₅) and transferred to autosampler vials. Of the mixture, 10 µL were injected directly into the LC-MS system, which was equipped with a 5-µm Hypercarb analytic column (100 × 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 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 (52, 66).

EtG in plasma

Measurement of EtG in plasma was performed by a negative ion electrospray LC-MS method (52, 66). The LC-MS system used for *Study IV* was an Agilent 1100 series LC-MS. All plasma samples, controls and standards were stored at -20°C until use. Before analysis, a 200-µL aliquot of plasma was mixed with 1.0 mL methanol containing internal standard and centrifuged at 14,000 g for 10 min. The supernatant was evaporated to dryness under a stream of nitrogen and the final content dissolved in 100 µL of de-ionized water. A 10-µL aliquot was injected directly into the LC-MS system, which was equipped with a 5-µm Hypercarb analytic column (100 × 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 SIM of m/z 221 for EtG, m/z 226. A calibration curve for EtG prepared by serial dilution with EtG negative plasma samples was linear ($r^2 = 0.994$, $p < 0.0001$) in concentration range 0.1–100 mg/L. The EtG concentration of unknown samples was determined from the peak-area ratios between EtG and EtG-D5 by reference to the calibration curve. The LOQ was ~0.15 mg/L EtG.

5HTOL

Urinary 5HTOL was determined by GC–MS using an HP 5972 (Hewlett Packard, Palo Alto, CA) (84). 5HTOL is mainly excreted in conjugated form with glucuronic acid (135, 136). Before analysis, the urine samples were spiked with an internal standard (deuterium-labelled 5HTOL; 5HTOL-d₄) and incubated at 37°C for 1 h with β -glucuronidase (β -D Glucuronoside glucuronosohydrolase, EC 3.2.1.31 from *E. coli*), resulting in complete hydrolysis of the 5HTOL glucuronide. The free 5HTOL was then extracted with diethyl ether and finally derivatized. Separation was achieved on a DB1701 capillary GC column (30 m \times 0.25 mm i.d.; J&W Scientific, Folsom, CA). GC-MS analysis was performed with 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 (137). 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 \times 4.0 mm i.d.). The mobile phase consisted of 105 mmol/L citric acid, 12 mL/L methanol, 50 μ mol/L EDTA and 25 μ mol/L sodium octylsulfate (pH 2.2) and the flow rate was 1 mL/min. The concentration of 5HIAA was calculated from the peak-area ratios to the internal standard by reference to a calibration curve. The LOQ of the method was ~1 μ mol/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 (138). In *Study I*, creatinine was determined by the routine Jaffé reaction, where creatinine reacts with picric acid under alkaline conditions to form a red-coloured 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 coloured 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.

10. RESULTS AND DISCUSSION

10.1. Study I

According to self-report, all participants taking part in this study had abstained from alcohol 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 5A). The EtG concentration also started to increase and was detectable already in the first urine collection after ethanol intake at 1 h (Figure 5B). 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 5A), 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 5B). 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 5C). 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 and later observations (35, 139, 140). 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. This has also been confirmed in later studies (59, 69, 70).

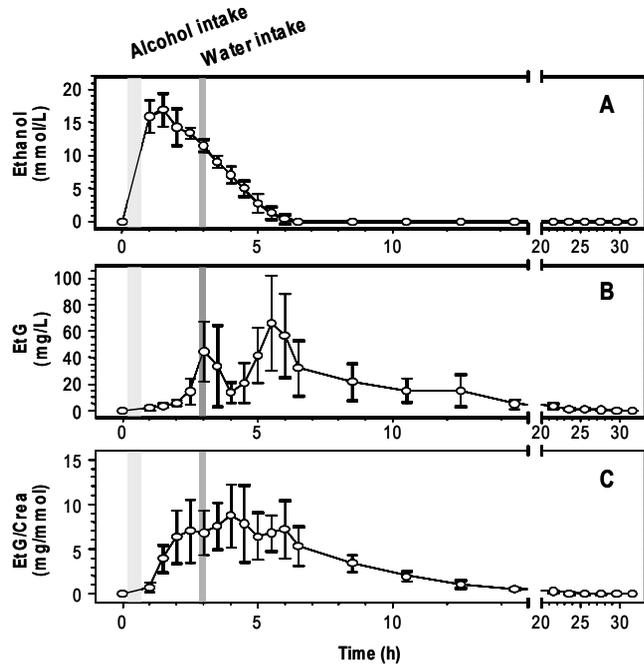


Figure 5. Time-course of diuresis and the urinary concentrations of ethanol (A) and EtG (B), and the urinary EtG/creatinine ratio (C), after six healthy volunteers drank 594–986 mL export beer (corresponding to 0.5 g/kg ethanol) in 30 min and 3 h later the same volume of water.

The interest in EtG as a biochemical marker of acute alcohol intake relates to it being a direct metabolite of ethanol detectable for several hours to some days longer than ethanol, implying both a high specificity and sensitivity for recent alcohol consumption (35-37, 68, 141-143). The high sensitivity of EtG was confirmed by the original observation that intake of a very small ethanol dose (~7 g) resulted in detectable EtG levels over at least 6 h (52, 144). Furthermore, following heavy drinking, EtG has been reported to be detectable for up to 4 days after the last ethanol intake (54, 143). EtG can also be detected in samples of hair, body fluids and tissues (55-60, 145, 146).

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 (48) but our results have later been

confirmed in other studies (59, 69). 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 and later observations (37, 53, 147), this did not influence the concentration of ethanol. Accordingly, it is possible to decrease the urinary concentration of EtG to fall below the LOQ 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 (148).

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 (138, 147, 149). However, even when EtG was expressed as a ratio to creatinine, there were considerable inter-individual variations in the excretions profiles, which were later confirmed by others (59, 69, 150). The individual variation could depend on factors such as differences in ethanol distribution and elimination, enzyme activities, enzyme induction, kidney disease, and polymorphism of the UGT enzymes (45, 47, 151).

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.

10.2. 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 6). 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) (134). 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.

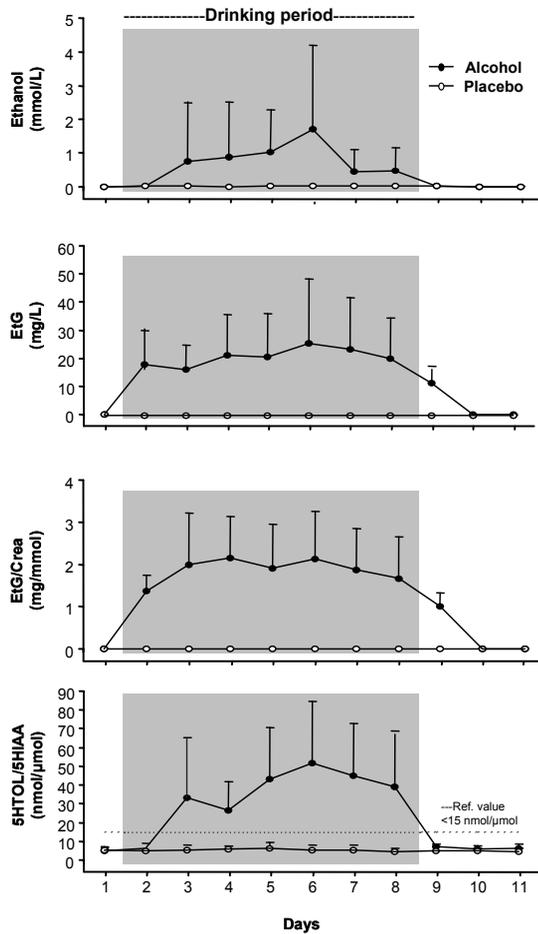


Figure 6. Urinary concentrations of ethanol and EtG, and the ratios of EtG/creatinine and 5HTOL/5HIAA, in first morning urine void samples collected during ethanol (0.4 g/kg twice daily, once in the morning and once in the evening) and placebo drinking periods.

An increased urinary ratio of 5HTOL/5HIAA has been used clinically for several years as a sensitive biochemical marker for recent alcohol consumption (33, 69, 70, 82, 86, 87, 141). More recent studies have demonstrated that analysis of EtG in urine can also be useful for this purpose (28, 35, 36, 152-154). 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 (35, 78). 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 (83, 155).

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 (52, 156).

In cases of prolonged heavy drinking, EtG has been reported to be detectable for up to 5 days after the last alcohol intake (54, 141, 143). This observation, together with other reports, suggested that EtG may accumulate in the body upon prolonged drinking (35, 54). However, the present study found no indication of EtG accumulation, nor a gradually increased 5HTOL/5HIAA ratio, upon repeated ethanol exposure of 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% (134). 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 night-time, 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 was 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. This has later been confirmed in other studies (69, 141).

10.3. 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 7). 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.

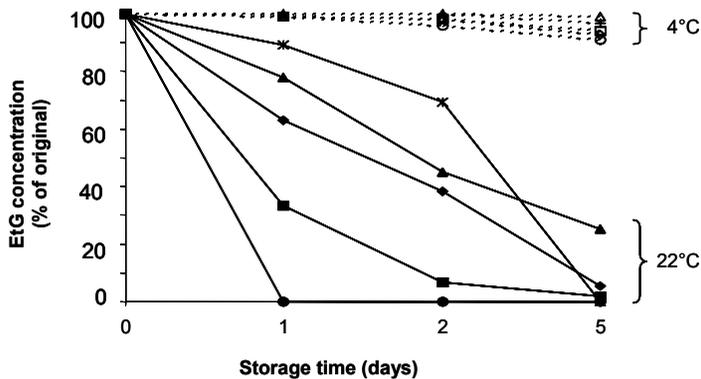


Figure 7. Effect of storage time and temperature on the hydrolysis of EtG by bacterial β -glucuronidase in clinical urine specimens containing *E. coli*.

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 2) caused hydrolysis of EtS after samples were supplemented with 1 mg/L EtS and stored at 22°C for 1–5 days.

Table 2. 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	Specimens causing hydrolysis of EtG ^a	Specimens causing hydrolysis of EtS ^a
	n	n (%)	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.

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 (66, 155). EtG and more recently EtS have thus been introduced as sensitive and specific biochemical markers of recent alcohol intake and appears to follow the same pattern of urinary excretion as EtG but exposure in lower concentration (37, 54, 67-70, 145, 153, 157). 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 (158). Sulfatase activity has

also been detected in many bacteria (159) but not in *E. coli* (160, 161) or only in very low amounts (162).

The present study demonstrated that bacterial β -glucuronidase, especially if samples are positive for *E. coli*, can hydrolyze EtG and cause false low or false-negative results. These results agree with previous studies showing that most *E. coli* strains possess β -glucuronidase activity (163-165) (*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 (164, 165). 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 (37, 66).

10.4. Study IV

This study compared the value of plasma EtG testing with the information about alcohol consumption obtained using the standard alcohol biomarkers GGT and CDT and the AUDIT questionnaire in an emergency department. Male patients (n = 81) diagnosed to be minimally injured and clinically non-intoxicated were screened about their alcohol consumption (amount and time of alcohol intake) by using the computerized AUDIT questionnaire and a paper-and-pencil assessment. Blood samples were collected for determination of ethanol, EtG (LC-MS) and GGT in plasma and %CDT in serum (%CDT immunoassay). Based on the AUDIT questionnaire 28% were classified at a risk for alcohol related problem (>8 points) and 63% as social drinkers

(1-7 points) and 9% as non-drinkers (0 points). Only 4% (3) of the patients showed a detectable ethanol concentration (0.01–0.07 g/L) but 38% (31) showed a detectable EtG in plasma (0.16–39.5 mg/L). In 9% (7) of the patients EtG was detectable in blood >24 h after calculated time for complete elimination, assuming an elimination rate of 0.15 g/kg/h and a distribution volume of $0.7 \times$ body weight and in 5% (4) patients EtG was detectable for >48 h afterwards, which indicated misreporting (Figure 8). A later study suggested that the suspected misreporting data could instead have been related to renal failure (151).

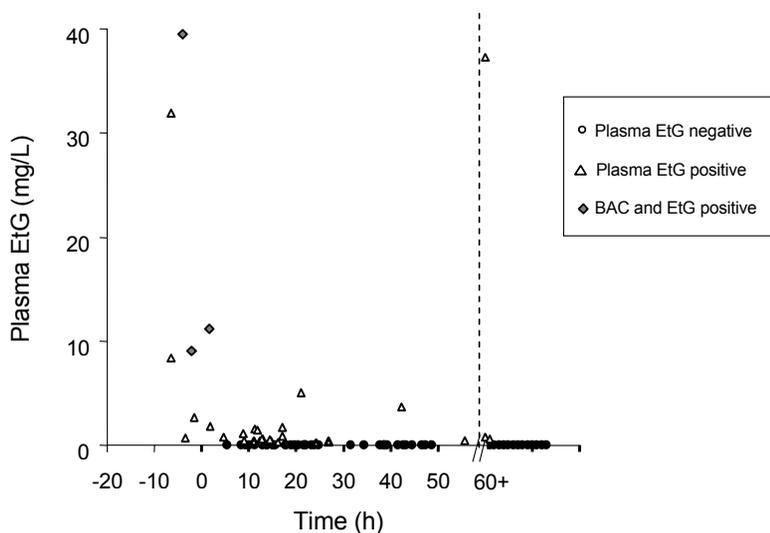


Figure 8. The time course of plasma EtG versus time to (negative values) or since (positive values) calculated completed ethanol elimination from the body, assuming an ethanol elimination rate of 0.15 g/kg/h.

EtG did not correlate with the long-term biomarkers %CDT or GGT, or the AUDIT results, but with the time since estimated completed ethanol elimination. Patient that appeared intoxicated at admission to the ED were excluded in this study. The findings that 4 of 7 patients that reported recent alcohol intake had no detectable ethanol which limited the value of ethanol testing in this setting. The high frequency (38%) of the patients being positive for EtG in plasma, confirmed that they had consumed alcohol within the past ~12–24 h (50, 140). A high alcohol intake is a common cause of trauma and patient with chronic and heavy alcohol consumption exhibit a higher incidence of morbidity and mortality following surgery (166), therefore it is important allow

intervention and reduction of alcohol-related harm (29, 129, 167, 168). This study employed plasma samples for testing EtG although EtG is detectable for considerable longer time in urine (50, 59, 140). In a later controlled study results showed that urinary EtG (C_{max}) were 160 times higher than blood (59). However it might not always be feasible to collect urine in the ED and most of the patients participating in this study were reluctant to leave urine sample whereas blood sampling was more tolerated. Even if blood is taken the detection time in plasma EtG is still longer than for ethanol but not as long as urinary EtG (59). EtG testing in blood was found useful in the ED as a way to detect recent drinking, even in cases of a negative ethanol test, and to confirm abstinence from alcohol. This plasma EtG is a sensitive and specific short-term biomarker provides valuable additional information about individual drinking habits and might also be helpful to identify an alcohol hangover.

10.5. Study V

This study was voluntary, anonymous and included urine sampling and a single-question form about recent drinking (“Did you drink any alcohol in the past 3 days – Yes/No?”). 24 patients (21 men and 3 women, aged 21–52 years) agreed to take part in this evaluation about recent drinking. The subjects were allocated to 3 sub-groups depending on the type of outpatient treatment program: Group *A* included 8 individuals (7 men and 1 woman) undergoing treatment mainly for alcohol dependence; Group *D* included 10 individuals (9 men and 1 woman) being treated for drug dependence (former heroin addicts); and Group *M* included 6 individuals (5 men and 1 woman) undergoing methadone maintenance therapy for opioid addiction. Each of the 24 patients provided 4–14 (mean 8.9, median 9) urine samples over a 2–8-week (mean 5.4, median 5) period, the total number of samples being 214 (Table 3). In 211 (98.6%) of the 214 cases, the anonymous self-report form about recent alcohol use was successfully completed. Altogether 26% of the urine samples from 12 of 24 patients tested positive for EtG (0.5–434 mg/L, mean = 47.1, median = 13.6) and/or EtS (range = 0.1–87 mg/L, mean = 10.8, median = 2.6). One patient (*D4*) (Table 3) was only positive for EtS (2.4–12.0 mg/L, mean = 5.8, median = 3.3) and these EtS results were confirmed by LC–MS/MS analysis (66). In 21 of the 211 self-reports collected from 11 patients, ingestion of alcoholic beverages was admitted in the 3-day period prior to urine sampling. In 87% of the 211 complete cases (both self-report and urinary data were available), the information provided by self-report agreed with the corresponding EtG and EtS results (true positives or true negatives) (Table 3).

Table 3. Frequency of recent alcohol consumption according to anonymous self-reports and urinary EtG and EtS measurement in outpatient treatment programs for alcohol and drug dependence.

Group and ID no. ^a	Sex (M/F)	Data collection (weeks)	Samples (N)	Self-reported drinking ^b / Urinary EtG and EtS results ^c					
				Pos/Pos	Neg/Neg	Pos/Neg	Neg/Pos	Missing/Pos	Missing/Neg
A1	F	5	12	1	11				
A2	M	5	9		9				
A3	M	5	10		7	1	2		
A4	M	6	10		10				
A5	M	5	7		7				
A6	M	5	8		8				
A7	M	3	4				4		
A8	M	2	4		4				
D1	M	7	10		10				
D2	M	7	12	10	2				
D3	F	6	9	3	4	2			
D4	M	8	9				8 ^d	1 ^d	
D5	M	6	10	9	1				
D6	M	6	9		6	1	2		
D7	M	8	14	1	11	2			
D8	M	4	11		11				
D9	M	8	12	4	7		1		
D10	M	5	7	3	4				
M1	M	5	10		8				2
M2	F	5	7		7				
M3	M	5	7		7				
M4	M	4	7		7				
M5	M	5	8	5		2	1		
M6	M	5	8		7	1			
<i>Total:</i>	<i>21M/3F</i>	<i>130</i>	<i>214</i>	<i>36</i>	<i>148</i>	<i>9</i>	<i>18</i>	<i>1</i>	<i>2</i>

^a Outpatients undergoing treatment programs for primarily (A) alcohol dependence or (D) drug dependence, or (M) opiate addicts participating in methadone maintenance therapy.

^b "Any intake of alcoholic beverages in the past 3 days – Yes/No?".

^c The reporting limits were > 0.5 mg/L for EtG and > 0.1 mg/L for EtS.

^d These urine samples were only positive for EtS.

In 9% of the cases EtG and EtS tested positive while this was not admitted by self-report and in another 4% of the cases recent drinking was reported but EtG and EtS were negative. There were significantly more positive self-reports of recent drinking when sampling occurred on Mondays (32.6%) compared with on Fridays (14.3%; $p = 0.0068$, Chi-square test). There was also a higher frequency of positive EtG and EtS findings in urine specimens collected on Mondays (32.2%) compared with on Fridays (21.5%) but this difference was not statistically significant ($p = 0.146$). The frequency of drinking (Figure 9) was significantly higher in the drug dependence group compared with the alcohol dependence group, both according to self-reports (34.3% vs. 3.1% of cases; $p < 0.0001$) and urinary EtG and EtS results (40.8% vs. 10.9%; $p = 0.0001$).

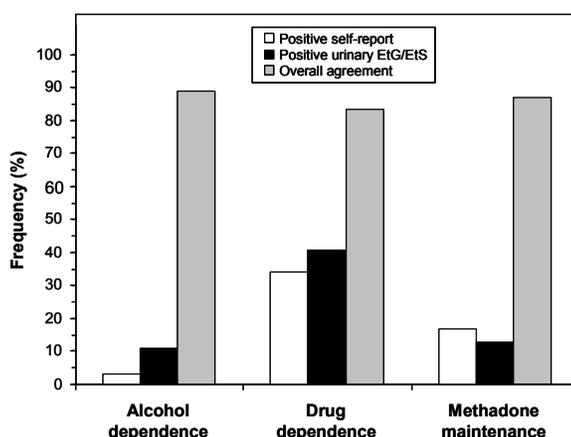


Figure 9. Indications of recent drinking based on anonymous self-report (any drinking in the past 3 days?) or a positive urinary EtG and EtS test in an outpatient treatment program for alcohol and drug dependence. “Overall agreement” is the total frequency of concordant results (the sum of results being positive for alcohol by both measures or negative by both measures). The reporting limit was 0.5 mg/L for EtG and 0.1 mg/L for EtS.

The frequency of drinking was also significantly higher in the drug dependence group compared with the methadone maintenance therapy group, according to urinary EtG and EtS (12.8%; $p = 0.0013$) but not to self-reports (17.8%; $p = 0.0672$). Furthermore, there was a significant difference in the frequency of self-reported drinking between the alcohol dependence and methadone maintenance therapy groups ($p = 0.0225$) but not according to urinary EtG and EtS ($p = 0.993$). Only 2 out of 10 (20%) patients in the drug dependence group abstained completely from drinking according to both

measures, compared with 5 of 8 (63%) in the alcohol dependence group and 3 of 6 (50%) in the methadone maintenance therapy group.

Alcohol represents a common problem among patients dependent on illicit drugs and heavy drinking is associated with an increased risk for relapse into drug use and discharge from treatment (169, 170), therefore monitoring of alcohol can be recommended for early detection. When new biomarkers are introduced in routine clinical use, the specificity for alcohol and the potential consequences for the patient of a false-positive or false-negative result need to be considered (171). A positive laboratory result taken as proof of relapse into alcohol and/or drug abuse might lead to discharge from treatment.

Several studies have provided data on the urinary detection times for EtG and EtS following intake of different alcohol doses, which represents important information in clinical practice (143). It should be considered that even consumption of a very small ethanol dose (<10 g), and use of ethanol-based products such as mouthwash (144) and hand sanitizers (172) may result in a detectable EtG level in urine (37, 52, 70). Furthermore, incorrect storage of infected samples implies a risk for false-positive as well as false-negative EtG results (150, 155, 173, 174), although this is seemingly a minor problem with EtS. Related to this, the cut-off used to distinguish between a positive and a negative result is always a critical issue. However, the risk for such problems was indicated to be low for the reporting limits applied for EtG (>0.5 mg/L) for EtS (>0.1 mg/L) in the present study (143). In this study the outcome was very high, with 99% of all self-report forms being completed successfully and even the corresponding EtG and EtS results agreed well (range for overall agreement = 83.5–89.1%). One major reason can be that the participation was voluntarily and did not influence their treatment in any way. If the self-reports had not been anonymous, these results would probably have been considerably lower (12, 13). The frequency of drinking was markedly lower among patients undergoing treatment for alcohol dependence compared with the other two groups. These results suggest that the patients in the treatment program focusing primarily on alcohol-related problems were more motivated to avoid drinking. Although this study based on a small sample size, these results suggest that urinary EtG and EtS testing is a valuable objective tool for detection of recent drinking during outpatient treatment. Biomonitoring of alcohol use may be important in any treatment program for alcohol and/or drug dependence.

10.6. Study VI

This study involved 56 patients (26 women and 30 men) diagnosed for alcohol dependence according to DSM-IV. The patients were recruited via advertisements in a local newspaper and instructed to participate in a double-blind study with either tablets of acamprosate (2 g/day) or placebo. They were told to abstain from drinking during the 3 week treatment period, beginning on the day they took their first dose. During the treatment period, patients made weekly return visits to the ward to fill out questionnaires about alcohol intake (TLFB) and to leave a urine sample on day 7, 14 and 22 (leaving urine sample at day 7 and 14 was optional). On day 22, they were also required perform a negative breath alcohol test. On the initial day of the study (day 0), a urine sample was obtained from 46 (82%) patients. Because leaving urine was optional on day 7 and 14, much fewer urine samples were collected on those days (52% and 61%, respectively), and these results were therefore excluded from the calculations. On the final study day (day 22), 47 (87%) patients delivered the mandatory urine sample. From 19 patients on acamprosate medication and 21 on placebo, a urine sample was obtained both on day 0 and 22.

On the first study day, 33 of the 46 patients (72%; 65% for acamprosate and 78% for placebo) tested positive for recent drinking according to EtG (LOQ >0.5 mg/L) and EtS (LOQ >0.1 mg/L). On the final day, the frequency of positive urine tests was significantly reduced to 30% among those randomized to acamprosate medication ($p = 0.0374$) and 33% for the placebo group ($p = 0.0050$) (Figure 10).

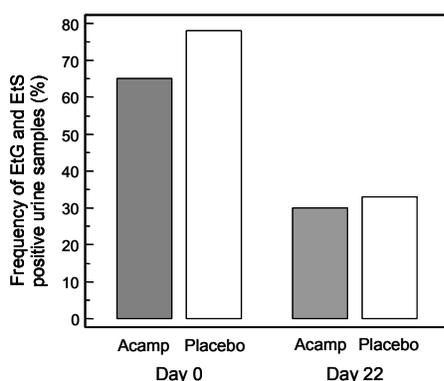


Figure 10. Frequency of urine samples positive for EtG and EtS in alcohol-dependent patients randomly allocated to 21 days of acamprosate medication (2 g/day) or placebo. Samples were collected on the initial (day 0) and final (day 22) day of the study.

However there were no significant differences between the treatment groups. When the results for both groups were combined, the urinary EtG and EtS concentrations on day 22 were significantly lower compared with on day 0 (Figure 11). For comparison all patients tested negative for ethanol in breath on the final day.

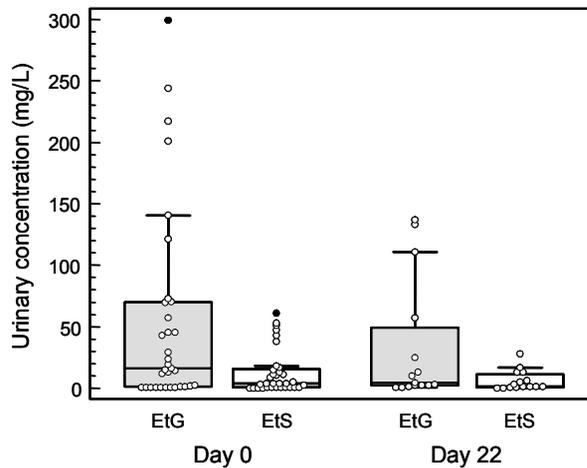


Figure 11. Box-and-whisker plot showing the distribution of urinary EtG and EtS concentrations on day 0 and 22 in samples collected from patients allocated to acamprosate medication or placebo. The Box-and-whisker plot shows the median, 25th and 75th percentiles, minimum and maximum values, and outside (○) and far out (●) values.

Altogether 63 urine samples from 41 patients (21 on acamprosate and 20 on placebo) of the totally 156 (40.4%) specimens collected within this study tested positive for EtG (range 0.50–300 mg/L, mean 44.6, median 15.2) and EtS (range 0.11–61.0 mg/L, mean 10.2, median 3.78). When separated by treatment group, there were 30 of 80 (36.5%) EtG and EtS positive samples in the patients on acamprosate and 33 of 76 (43.4%) positives in the placebo group.

In addition to the 63 EtG and EtS positive urine samples, another 13 (8.3%) contained measurable amounts (LOD for EtG <0.1 mg/L and EtS <0.05 mg/L) of both metabolites but at levels below the routine LOQ. Accordingly, based on urinary EtG and EtS testing, almost half (48.7%) of all specimens indicated recent drinking.

Based on self-report, in 26 of the 63 positive cases (41.3%) alcohol consumption was admitted (range 1–8 standard drinks, mean 4.3, i.e. ~50 g ethanol) on the previous day; in 19 of these drinking was also admitted 2 and/or 3 days back. In another 33 cases, alcohol consumption was only admitted 2 and/or 3 days back. Two patients denied any drinking over the past 3 days. Self-report data was missing for 2 subjects. In 28 cases where the patients reported alcohol consumption (range 1–8 drinks, mean 4.1 ~50 g ethanol) on the day before urine sampling, 26 (92.9%) urine samples tested positive for EtG and EtS. In the remaining cases where no drinking on the previous day was admitted, 35 (27.6%) samples were positive for EtG and EtS.

In the 93 urine samples that tested negative for EtG and EtS (<LOQ), there were no positive self-reports of drinking on the day prior to sampling, while in 15 cases alcohol consumption was admitted 2 and/or 3 days back.

Altogether there were 77 cases of self-reported drinking over the 3-days period prior to each sampling, ranging from 1–25 standard drinks (12–300 g ethanol, mean 7.3 drinks ~90 g ethanol). Overall the self-reported quantity of drinking over the past 3 days prior to urine sampling showed good correlation with the EtG and EtS concentrations (Figure 12).

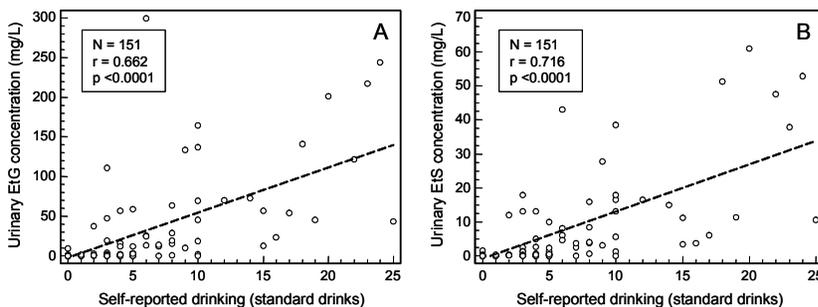


Figure 12. Correlation between self-reported drinking, expressed as number of standard drinks (12 g ethanol) over the past 3 days, and the corresponding urinary (A) EtG and (B) EtS concentrations.

This study population comprised treatment-seeking patients participating in a 3-weeks randomized double-blind study to determine the effect of acamprosate medication on alcohol-cue reactivity and alcohol priming (132). Although the patients were instructed to refrain from alcohol during the treatment period, and the goal of abstinence was further supported by counselling in connection with the return visits to the ward, the

results revealed that almost 50% of all urine samples tested positive for EtG and EtS. The EtG and EtS concentrations showed good correlation with the self-reported quantity of drinking over the past 3 days prior to urine sampling. However, it must be highlighted that evidence of drinking was not a ground for study discontinuation, except for a positive breath test on day 22. This could be a reason behind continued drinking during the treatment period and for the good reliability of the self-reports. This observation, together with that of other studies, suggests that there is a good correlation between self-report and the EtG and EtS results in cases where alcohol intake during treatment does not lead to any consequence (175). Other studies have shown that, when admitting alcohol use could have negative consequences to the individual, then the self-report information may be unreliable (13, 84).

In agreement with previous studies, the present results indicated a dose- and time-dependent sensitivity of urinary EtG and EtS as biomarkers for detection of recent drinking. It has been demonstrated that both metabolites can pick up ingestion of even very small amounts of alcohol for roughly 1 day afterwards, and larger doses for 2 or possibly 3 days (52, 59, 68, 69, 143, 176).

In cases when drinking was admitted in the day before sampling, 93% of the urines tested positive for EtG and EtS. In addition, the EtG and EtS biomarker also detected a large number (28%) of positive cases when prior drinking had been denied. Overall, however, the results indicated that both the frequency and amount of drinking were significantly reduced at the end of the treatment period compared with the starting values. In this respect, acamprosate apparently offered no advantage over placebo. This is in line with earlier trials demonstrating an effect of acamprosate medication over placebo typically after a longer treatment period.

Taken together, the present results highlight the value of using sensitive and specific alcohol biomarkers to detect alcohol consumption during outpatient treatment of patients with alcohol-related problems, and as objective outcome measures when evaluating new treatment strategies and pharmacotherapies, and not to rely solely on patient self-reports.

11. 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 following alcohol intake. 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. EtG testing in plasma was found to be useful in the ED as a way to detect recent drinking, even in cases of a negative ethanol test and to confirm abstinence from alcohol. The plasma EtG result also provide valuable additional information about individual drinking habits and might be helpful to identify an alcohol hangover. The EtG and EtS testing might be a helpful tool for detecting recent drinking in any treatment program for alcohol and/or drug dependence. EtG and EtS may also be objective outcome measures when evaluating new treatment strategies and pharmacotherapies. 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. This sensitive and specific alcohol biomarker may be valuable for objective detection of recent alcohol consumption and relapse drinking, a helpful tool in interventions that may reduce a hazardous/harmful alcohol use, in hospital settings, and as an objective outcome measures when evaluating new treatment strategies and pharmacotherapies.

12. ACKNOWLEDGEMENTS

This work would not have been possible without the help and support of many great people.

I wish to express my sincere thanks to:

Anders Helander, my supervisor and boss, for all your help and for inviting me into the world of alcohol research and biochemical markers. Thanks for my time at the Alcohol laboratory.

My co-authors *from; clinical pharmacology Nikolai Stephanson and Olof Beck; Finland Taisto Sarkola and Peter Eriksson; Germany Tim Neumann, Tilly Holzmann, Bruno Neuer, Edith Weiß-Gerlach, Christian Müller and Claudia Spies; Maria Addiction Center Annette Voltaire Carlsson and Kristina Hillgren; clinical neuroscience KI Anders Hammarberg and Johan Franck.*

My colleagues at the Alcohol laboratory *Yufang Zheng and Naama Kenan* Good luck!

My former colleagues *Cattis Löwenmo, Tina Burenius, Marie Olsson and Tina Andersson* in the beginning of Alcohol laboratory. This is it!!!

Margareta Some for your friendship and all your knowledge, help and support during the time from day one when we began at Alcohol laboratory till the end!

The boys *Jonas Bergström and Kristian Björnstad* that ended the Ph.D studies at Alcohol laboratory. I just say Barcelona lucky you!!!

Bim Linderholm and Marie Haegerstrand-Björkman “I miss you” *Guido Stichtenoth, Andrea Calkovska, Andreas Almlen and Tore Curstedt* from *SurfactantLab, Kajsa Bohlin, Eva Henckel, Jan Svensson* and all *coffee-room friends* for created the atmosphere so great.

My new room mates *Marjan Shafaati, Tina Kannisto, Xiao-Li Hu, Veronika Tillander* for making me feel so comfortable when I comes to the PhD room and to *Jenny Bernström* for taking care of everything!

To all my new colleagues at *Huddinge 2* for taking care of me and all your support – you all mean a lot to me and I look forward to spend more time with you!!!

To my brother *Janne* for always being there!

My nephews *Joakim, Fredrik och Johan* ☺

My parents *Anita & Seppo* for always encouraging and believing in me ♥

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