Utility of urinary ethyl glucuronide analysis in post-mortem toxicology when investigating alcohol-related deaths

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ABSTRACT

Use and abuse of alcohol are common findings when unnatural deaths are investigated as evidenced by high blood- and urine- alcohol concentrations (BAC and UAC) at autopsy. Because ethanol is metabolized in the liver until the time of death, the autopsy BAC or UAC might be negative even though the deceased had consumed alcohol in the immediate ante-mortem period. Analysis of the non-oxidative metabolite of ethanol [ethyl glucuronide (EtG)] offers a more sensitive test of recent drinking. In this paper, we determined the concentrations of ethanol and EtG in urine samples from 972 consecutive forensic autopsies. In 425 cases (44%) both EtG and ethanol were positive, which supports ante-mortem drinking. In 342 cases (35%), both EtG and ethanol was negative, which speaks against any consumption of alcohol just before death. In 181 cases, ethanol was negative in urine (<0.2 g/kg), whereas EtG was positive (>0.5 mg/L), which points towards ingestion of alcohol some time before death. In these cases, mean and median concentrations of EtG were 53.2 mg/L and 23.7 mg/L, respectively, although there was no mention of alcohol on 131 of the death certificates. Alcohol was mentioned on death certificates as an underlying or immediate cause of death or a contributing factor in 435 (45%) cases, which rose to 566 (58%) cases when positive EtG results were included. This article demonstrates the usefulness of EtG analysis in routine post-mortem toxicology when ante-mortem drinking and alcohol-related deaths are investigated.

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1. Introduction

Over-consumption of alcohol and drunkenness are contributing factors in many unnatural deaths, such as alcohol-attributable diseases, acute poisonings, work-related and traffic accidents, suicides and homicides [1–3]. Mortality rates associated with use and abuse of alcohol are higher in Finland (population 5.5 million) compared with the other Nordic Countries [4,5]. Between 2004 and 2009, acute alcohol poisonings were reported on death certificates in 9 of 100,000 inhabitants [6]. Moreover, forensic autopsy rates are much higher in Finland compared with other nations, which gives a good opportunity to study the role of alcohol and drug abuse in unnatural deaths.

Lahti et al. [7] reviewed the characteristics of unnatural deaths in Finland reporting that in 87% of cases alcohol use and/or abuse was considered one of the contributing factor. The mean autopsy blood alcohol concentration (BAC) in these deaths was 2.2 g/kg. In the same study, alcohol poisoning was the principal cause of death in 27% of all cases and the mean BAC was 2.9 g/kg. In 20% of all unnatural deaths, alcohol was the only or the most important psychoactive substance identified in post-mortem blood and mean BAC in these cases was 3.3 g/kg [7].

Ethanol is the substance most often encountered in post-mortem toxicology and in the present study positive findings were reported if the concentration exceeded 0.2 g/kg [8]. However, through metabolism during life, a negative toxicology report does not preclude ante-mortem ingestion of ethanol. Analysis of ethyl glucuronide (EtG), the non-oxidative metabolite of ethanol, furnishes a more sensitive test of recent drinking because EtG takes longer to leave the body compared with ethanol [9]. After subjects drank a small dose of alcohol (0.5 g/kg), ethanol was measurable in urine for 6.5 h, whereas EtG was measurable for 23–32 h [10]. In alcoholics during detoxification, EtG was measurable in urine for 30–110 h (median 66 h) longer than ethanol [11].

This paper reports the utility of EtG in epidemiological studies of alcohol-related deaths beyond individual cases and case series.
The urinary concentrations of EtG were determined by DRI–EtG immunoassay in 972 consecutive forensic autopsies and positive ethanol and EtG results were evaluated in relation to whether alcohol use and/or abuse was mentioned on the death certificates.

2. Materials and methods

2.1. Urine samples

Urine samples were obtained from the bladder in connection with routine post-mortem examinations done by forensic pathologists in Finland. Sodium fluoride (≈1% w/v) was added to urine as a preservative and enzyme inhibitor to prevent microbial processes that might generate ethanol after sampling. The specimens of urine were stored at +4 °C until they were analyzed and the analytical toxicology work was completed within 12 days after the autopsy. The time from death to autopsy averaged six days and corpses were stored in a refrigerated state after arrival at the morgue.

Between January 2011 and April 2011, urine samples from a total of 972 routine forensic autopsies were subjected to determination of EtG, although the results of analysis were not disclosed to the pathologists when cause and manner of death were decided. Urine was collected from one healthy volunteer, who had abstained from alcohol for several days and this was verified negative for ethanol and EtG and used to dilute post-mortem urine specimens if the concentrations of EtG were unusually high.

2.2. Reagents

All the materials and reagents were purchased from Thermo Scientific (Passau, Germany). The DRI® immunoassay for EtG contained antibody/substrate reagent (reagent A) and enzyme conjugate reagent (reagent E). Reagent A comprised mouse monoclonal anti-ethyl glucuronide antibody, glucose-6-phosphate (G6P), and nicotinamide adenine dinucleotide (NAD+) in Tris buffer. Reagent E comprised ethyl glucuronide derivative labeled with glucose-6-phosphate dehydrogenase (G6PDH) in Tris buffer.

DRI® ethyl glucuronide calibrators (negative, 0.1, 0.5, 1.0, and 2.0 mg/L) were used to calibrate the assay and DRI® ethyl glucuronide controls (0.375, 0.652, and 0.750 mg/L) were used to validate the calibration. The calibrators and controls were made from blank human urine spiked with EtG. The reagents and the calibrators and controls contained sodium azide as a preservative. Water for priming the analyzer and cleaning the injection needle between every injection was purified with a Milli-Q Integral 5 (Millipore, Bedford, MA, USA).

2.3. Analysis of EtG and ethanol in urine

The immunoassay was done with a Thermo Scientific CDX 90 analyzer, which was used according to the manufacturer’s instructions. Urine samples were centrifuged before analysis and ~200 μL was placed in a sample cup and inserted into the analyzer. Calibration of the instrument was verified by analysis of three EtG control samples of known concentration. The homogenous and competitive assay makes use of specific antibodies that detect EtG and cross-reactivity with other glucuronides was negligible.

The immunoassay principle is well known and target drug labeled with glucose-6-phosphate dehydrogenase (G6PDH) competes with unlabelled drug contained in the urine sample for a fixed amount of antibody binding sites. When urine does not contain any EtG, the specific antibody binds the drug labeled with G6PDH and enzyme activity decreases. When EtG is present in urine, the active enzyme (G6PDH) converts NAD+ into NADH resulting in an absorbance change, which is monitored at 340 nm.

Urine samples were considered positive for EtG when change in absorbance was equal to or greater than that obtained for the calibrator (EtG = 0.5 mg/L). The assay was linear up to EtG concentration of 2 mg/L and samples with higher concentrations were diluted with blank urine (maximum dilution 1/200) and re-assayed.

Ethanol was determined in urine samples by dual-column headspace gas chromatography (HS–GC) after an aliquot (100 μL) of the specimen was diluted with 2-butanol as internal standard. This HS–GC procedure has been used in our laboratory for many years and gives accurate, precise and specific results. An ethanol cut-off concentration of 0.2 g/kg was used to report positive urine samples.

2.4. Unnatural death investigations

Sudden or unexpected deaths in Finland are investigated by the police authorities in collaboration with a forensic pathologist, who perform an autopsy and also consider all other relevant information before reaching a conclusion about the cause and manner of death. Deaths without known diseases, or when a person dies in a work-related or traffic-related accident, fatalities associated with violent crimes, suicide by various means, acute poisonings, medical procedure, etc. are all examples of sudden and unnatural deaths.

Official statistics show that 50,585 deaths occurred in Finland in 2011 and a medico-legal autopsy was performed in 10,602 cases (21%). A complete toxicological analysis was done in 6661 cases (13.2% of all deaths). The analysis of EtG in urine is usually ordered by the responsible forensic pathologist although for purposes of the present study such an analysis was done in 972 consecutive cases.

3. Results

3.1. Subject demographics

The median age of all the 972 deaths was 59 years and there was a clear predominance of males over females (84%–16%). Demographics of the deceased are shown in Table 1 in relation to whether ethanol and/or EtG were positive or negative in the urine specimens.

3.2. Concentrations of ethanol and EtG in urine

The concentrations of ethanol in urine ranged from 0.2 to 6.7 g/kg (median 2.2 g/kg) and these results are shown in Table 2 in relation to mean and median concentrations of EtG for increasing levels of ethanol.

The frequency distribution of EtG concentrations in urine for all cases (N=972) is shown in Fig. 1 when the upper limit of quantitation by the immunoassay was 400 mg/L.

3.3. Mention of alcohol on death certificates

Death certificates from 972 consecutive post-mortem were available for scrutiny (Table 3). These certificates showed that alcohol use or abuse was mentioned in 435 cases (45%) compared with 537 cases (55%) without any mention of alcohol. Both ethanol and EtG were positive in urine samples from 425 cases (44%) and alcohol was mentioned in 361 of these (85%).

In 342 cases (35%), both urinary EtG and ethanol were negative and there was no mention of alcohol on 94% of these death certificates. However, in 24 urine samples (2%), ethanol was positive and the concentrations ranged from 0.2 to 2.7 g/kg.
(median 0.59 g/kg), even though EtG was negative (<0.5 mg/L). The cadavers in 11 out of the 24 cases were gutted making it more likely that ethanol had been produced after death by fermentation processes. Three of the deceased suffered from diabetes and probably had glucose in urine, thus providing a good substrate for post-mortem synthesis of ethanol.

When four methanol poisoning deaths were omitted, urine samples from the remaining 177 cases (18%) were negative for ethanol (<0.2 g/kg), although EtG was positive (>0.5 mg/L) at mean and median concentrations of 53.2 mg/L and 23.7 mg/L, respectively. Alcohol was not mentioned on 131 of these death certificates, although positive EtG makes it very likely that the deceased had been drinking in the immediate ante-mortem period.

4. Discussion

Research on ethyl glucuronide as a trace non-oxidative metabolite of ethanol increased appreciably after 1995 when an improved GC–MS method of analysis was presented [13]. During the past 20 years, a remarkable number of research articles and reviews have appeared dealing with various aspects of EtG analysis in forensic and clinical toxicology [14]. Methods have been described for the analysis of EtG in blood, urine, vitreous humor, and hair specimens by both GC–MS and a more practical immunoassay screening method [15,16]. The definitive method of EtG analysis involves liquid chromatography–mass spectrometry (LC–MS/MS) with deuterium labeled internal standard [17–19].

Böttcher et al. [15] described an immunoassay method (DRI–EtG–EIA) for analysis of EtG in urine, which was ideal for processing large numbers of samples [20]. Moreover, the screening results agreed well with analysis of EtG by an LC–MS/MS for a wide range of concentrations of the ethanol conjugate in samples from living subjects [11] and post-mortem cases [20]. The upper limit of quantitation of the EtG immunoassay used in the present post-mortem study was 400 mg/L.

Pharmacokinetic studies show that concentration-time profiles of ethanol and EtG in blood and urine are shifted in time [21]. The peak concentrations of EtG are reached 1–2 h later than the peak concentration of ethanol and the EtG levels are roughly 1000 times less than the ethanol concentration [22]. More recently, considerable interest has developed in the analysis of EtG on hair strands as a way to monitor abstinence in people not permitted to drink alcohol, although positive results should be interpreted with caution, owing to risk of contamination from extraneous non-beverage sources of ethanol and EtG [23,24].

Positive blood and/or urine EtG results verify metabolism of ethanol in the body during life, which makes it more likely that the deceased person had consumed alcoholic beverages [25]. However, it seems that in people with urinary tract infections, small amounts of EtG might be produced in the sampling tubes by the action of bacteria [26]. Furthermore, if a person died shortly after drinking a bolus dose of ethanol, the urine sample collected at autopsy might be negative for EtG but positive for ethanol. This could be explained if there was a pool of alcohol-free urine in the bladder before drinking alcohol and considering the fact that it takes about 15 min before measurable amounts of EtG which are detectable in bladder urine [21].

To determine cause and manner of death in forensic casework, pathologists consider all available information including (i) autopsy findings and histopathology, (ii) discoveries at the death scene and (iii) analytical toxicology results [27]. In the present material death certificates, 435 out of 972 autopsies (45%) mentioned alcohol, either as the cause of death (underlying or immediate) or as a contributing factor. This number increased by

### Table 1
Demographics of the deceased in 972 consecutive forensic autopsies in Finland arranged according to whether ethanol and/or ethyl glucuronide (EtG) were positive or negative in urine samples.

<table>
<thead>
<tr>
<th>Results of ethanol and EtG analysis in urine</th>
<th>N (%)</th>
<th>Age, y (mean ± SD)</th>
<th>Gender, N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol and EtG positive</td>
<td>425 (44)</td>
<td>55 ± 14</td>
<td>Male: 380 (89)¹</td>
</tr>
<tr>
<td>Ethanol and EtG negative</td>
<td>342 (35)</td>
<td>61 ± 19</td>
<td>Male: 250 (73)²</td>
</tr>
<tr>
<td>Ethanol positive, EtG negative</td>
<td>24 (2)</td>
<td>65 ± 17</td>
<td>Male: 19 (79)³</td>
</tr>
<tr>
<td>Ethanol negative, EtG positive</td>
<td>181 (19)</td>
<td>56 ± 14</td>
<td>Male: 167 (92)³</td>
</tr>
</tbody>
</table>

¹ Significantly older than all other age groups (p < 0.001).
² Significantly more males than females in all age groups (p < 0.001).

### Table 2
Mean and median concentrations of ethyl glucuronide (EtG) in urine samples from consecutive autopsies arranged after increasing concentration of urinary ethanol. EtG analysis was done by an immunoassay method with an upper limit of quantitation of 400 mg/L.

<table>
<thead>
<tr>
<th>Urinary ethanol (g/kg)</th>
<th>N</th>
<th>Urinary EtG, mg/L (mean (median))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20–0.5</td>
<td>49</td>
<td>116 (90)</td>
</tr>
<tr>
<td>0.51–1.0</td>
<td>48</td>
<td>205 (194)</td>
</tr>
<tr>
<td>1.01–1.5</td>
<td>38</td>
<td>178 (135)</td>
</tr>
<tr>
<td>1.51–2.0</td>
<td>44</td>
<td>210 (153)</td>
</tr>
<tr>
<td>2.01–2.5</td>
<td>45</td>
<td>197 (149)</td>
</tr>
<tr>
<td>2.51–3.0</td>
<td>53</td>
<td>245 (266)</td>
</tr>
<tr>
<td>3.01–3.5</td>
<td>51</td>
<td>257 (303)</td>
</tr>
<tr>
<td>3.51–4.0</td>
<td>38</td>
<td>254 (261)</td>
</tr>
<tr>
<td>4.01–4.5</td>
<td>27</td>
<td>299 (325)</td>
</tr>
<tr>
<td>4.51–5.0</td>
<td>19</td>
<td>311 (400)</td>
</tr>
<tr>
<td>&gt;5.0</td>
<td>13</td>
<td>279 (291)</td>
</tr>
</tbody>
</table>

![Fig. 1](image.png)  
**Fig. 1.** Frequency distribution of the concentrations of ethyl glucuronide (EtG) in urine samples from 972 consecutive forensic autopsies. EtG analysis was done by an immunoassay method with an upper limit of quantitation of 400 mg/L.
Table 3
Urinary ethanol and ethyl glucuronide (EtG) results from 972 consecutive forensic autopsies arranged according to whether alcohol use and/or abuse was mentioned on the death certificates.

<table>
<thead>
<tr>
<th>Alcohol mentioned on death certificate</th>
<th>Both EtG and ethanol negative</th>
<th>Both EtG and ethanol positive</th>
<th>Ethanol positive, EtG negative</th>
<th>EtG positive, ethanol negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes (N=435) 45%</td>
<td>19 (4.4%)</td>
<td>361 (83%)</td>
<td>5 (1.1%)</td>
<td>50 (11.5%) a</td>
</tr>
<tr>
<td>No (N=537) 55%</td>
<td>323 (60%)</td>
<td>64 (12%)</td>
<td>19 (4.0%)</td>
<td>131 (24%)</td>
</tr>
<tr>
<td>All (N=972) 100%</td>
<td>342 (35%)</td>
<td>425 (44%)</td>
<td>24 (2%)</td>
<td>181 (19%)</td>
</tr>
</tbody>
</table>

a Includes 4 methanol poisoning deaths and one ante-mortem sample.

almost one third to 566 (58%) when N = 131 urine samples that were positive for EtG but negative for ethanol were included. This supports the analysis of EtG in post-mortem toxicology to give evidence of alcohol consumption in the immediate ante-mortem period.

It is widely known that alcohol can be produced in the body after death, especially in traumatic deaths and when corpses are at ambient temperatures for long periods [28]. Within 24 h after death, the tissue of the gut undergoes self-digestion by the action of enzymes (autolysis) and the bacteria spread into the vascular system [29]. Many types of bacteria or yeasts are capable of transforming sugars and other substrates (e.g. amino acids and lactate) into ethanol by fermentation processes [8]. Post-mortem synthesis of ethanol is exaggerated in obesity and in people with poorly treated diabetes, who might have glucose in urine samples at the time of death [30]. Deaths involving major disruptive trauma or burns (traffic or plane crash) also facilitate a more rapid decomposition with spread of bacteria heightening the risk of microbial synthesis of ethanol [31].

A good practice in post-mortem toxicology is to analyze ethanol in alternative specimens (eye–fluid, bladder urine or cerebrospinal fluid) and compare results with post-mortem BAC level [32]. Finding the expected blood-to-body fluid concentration ratios of ethanol helps to support the notion of ante-mortem drinking [8]. The delayed elimination kinetics of EtG compared with ethanol makes analysis of the conjugate a more sensitive test of recent drinking as shown by results in Table 3 [25]. In certain forensic cases, especially when the corpse is decomposed, analysis of ethyl sulfate is recommended because this ethanol conjugate is seemingly more stable in blood and body tissues after death [33,34].

The usefulness of EtG analysis in post-mortem toxicology was seen during investigation into the death of a 7-month-old baby boy. Autopsy blood was taken from the thoracic cavity and a sample preserved with sodium fluoride contained 0.33 g/kg ethanol and another preserved with EDTA contained 0.55 g/kg ethanol. The concentration of ethanol in bladder urine (with fluoride preservative) was 0.20 g/kg. The police initially suspected that someone had given alcohol to the baby but a negative urinary EtG (<0.5 mg/L) made it more likely that microbial synthesis of ethanol had occurred after death. As a further proof, a small volume of femoral blood (with NaF preservative) was negative for ethanol (<0.2 g/kg).

In deaths attributed to alcoholic ketoacidosis, the concentrations of ethanol in body fluids are often negative although the deceased was known to have a problem with alcohol and fatty liver was seen at autopsy [35]. Under these circumstances, analysis of EtG is recommended because it furnishes a wider detection window to confirm ante-mortem drinking. Analysis of EtG is also useful when infant deaths are investigated as described above and in the literature [36].

When deaths in people engaged in safety-sensitive work are investigated, the question of alcohol consumption becomes very important and analysis of EtG serves as a useful biomarker of recent drinking [14]. This might be relevant in connection with investigations of public transport crashes (train, boat, and plane) in which the driver or pilot was killed. Finding a negative urinary EtG and positive BAC or UAC raises a suspicion that ethanol, at least in part, was synthesized after death and was therefore a post-mortem artifact [36].

In the present post-mortem study, the mean and median UAC was 2.3 g/kg and 2.2 g/kg, respectively and 13% of urines contained more than 400 mg/L EtG (Fig. 1). These high concentrations of EtG are in good agreement with a study done in drinking drivers (N = 100) with mean UAC of 2.53 g/kg and when mean and median urinary EtG concentrations were 292 mg/L and 171 mg/L, respectively [37].

Alcohol-related disease was identified as an important factor on 22 death certificates and an additional 24 certificates mentioned death as a consequence of the alcohol withdrawal syndrome. In these 46 cases, ethanol was negative whereas EtG was verified positive in urine, which supports ante-mortem ingestion of alcoholic beverages. In 131 deaths, EtG was positive in urine but ethanol was negative and death certificates contained no mention of alcohol use or abuse (Table 3). The positive EtG results speak towards consumption of alcohol in the immediate ante-mortem period, and this information is otherwise not available to the pathologist.

Declaration

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