Comparison of Ethyl Glucuronide and Carbohydrate-Deficient Transferrin in Different Body Fluids for Post-mortem Identification of Alcohol Use
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Abstract — Aims: Alcohol abuse is a major risk factor for premature death. Confirming the role of alcohol consumption in cause-of-death investigations has, however, remained difficult, due to lack of reliable biomarkers. Methods: We compared ethyl glucuronide (EtG) and carbohydrate-deficient transferrin (CDT) assays from serum, urine, cerebrospinal fluid and vitreous humor in a forensic autopsy population with either a positive (n = 38) or negative (n = 22) history of alcohol abuse based on detailed medical and police records and forensic toxicological investigations. Results: A positive blood alcohol concentration (median 1.15‰, range 0–3.3‰) was found in 26/38 (68%) of the cases with a documented history of alcohol abuse. EtG concentrations (mean ± SD) in urine (339 ± 389 mg/l, P < 0.001), vitreous humor (4.2 ± 4.8 mg/l, P < 0.001), serum (6.9 ± 8.9 mg/l, P < 0.01) and cerebrospinal fluid (1.7 ± 2.7 mg/l, P < 0.01) were significantly higher among the cases with a positive history of alcohol use than those in the alcohol-history negative group, whereas in corresponding comparisons CDT was significantly different only in cerebrospinal fluid (4.3 ± 2.1 vs. 2.3 ± 0.6‰, P < 0.05). The highest sensitivities (92%) in detecting ante-mortem alcohol use were obtained for urine and vitreous humor EtG assays. Conclusion: Our data indicate that measurements of EtG in urine or vitreous humor show the highest diagnostic accuracies in post-mortem investigations of excessive alcohol consumption and can be recommended for routine applications.

INTRODUCTION
Alcohol abuse plays a major role in deaths resulting not only from accidents, suicides and crimes of violence but also from a wide variety of chronic diseases (Kugelberg and Jones, 2007; Rainio et al., 2008a). However, due to the lack of sensitive and specific biomarkers, assessment of excessive alcohol consumption in cause-of-death investigations has remained difficult. Pathological findings are rarely specific and background information is often inconclusive (Kugelberg and Jones, 2007; Rainio et al., 2008a). Measurement of the blood alcohol concentration (BAC) may confirm cases involving acute intoxication, but the use is limited to situations when ethanol is still present in the body and about half of the alcoholics have been estimated to die with a negative BAC (Sadler et al., 1996).

Although several different biomarkers have been developed during the past decades for the detection of excessive alcohol consumption (Niemelä, 2007), studies in forensic materials are limited (Helander et al., 1992; Sadler et al., 1996; Simomet et al., 1999; Osuna et al., 2000; Berkowicz et al., 2003; Rainio et al., 2008a,b). Carbohydrate-deficient transferrin (CDT) has been the most widely used test, although it is known to suffer from a lack of sensitivity (Sadler et al., 1996; Berkowicz et al., 2003; Hietala et al., 2006; Rainio et al., 2008a). CDT levels typically increase after continuous heavy drinking, such as prolonged consumption exceeding ~50–80 g ethanol per day on average, whereas occasional binge drinking will not be detected (Lesch et al., 1996; Schellenberg et al., 2005; Hietala et al., 2006).

Recent studies have shown that ethyl glucuronide (EtG), a direct conjugated minor metabolite of ethanol, is present in biological samples following ethanol ingestion (Schmitt et al., 1995; Alt et al., 2000; Wurst et al., 2000; Schloegl et al., 2006; Appenzeller et al., 2007; Neumann et al., 2008; Helander et al., 2009a). In urine, EtG remains detectable for several days after ethanol has been eliminated from the body and it therefore shows promise also as a “medium-term” biomarker of alcohol consumption (Wurst et al., 2000, 2003; Helander et al., 2009a; Hösíeth et al., 2010). It has been suggested that EtG may also be useful in forensic applications (Keten et al., 2009; Thierauf et al., 2011). However, the risks for post-sampling errors and sample stability need special consideration and there may be significant differences in the biochemical characteristics of different sample types (Harper, 1989; O’Neal and Poklis, 1996; De Martinis et al., 2006, Helander et al., 2007). Moreover, as yet there are no generally accepted cut-offs for the diagnostic use of biomarkers in forensic sample materials.

In order to shed further light on the practical use of alcohol biomarkers in cause-of-death investigations, the present work compared the diagnostic characteristics of immunological assays for EtG and CDT from serum, urine, cerebrospinal fluid and vitreous humor collected at autopsy from individuals with or without a history of alcohol abuse.

MATERIALS AND METHODS
Study material
Samples from 60 cadavers (48 males and 12 females, mean age 62 ± 15 years) were collected during autopsies at the Institute of Forensic Medicine of Tampere University, Finland. Cases were included into the study in a consecutive manner with special emphasis on the records of excessive alcohol use. Documentation of alcohol use was based on data collected separately from police reports (investigations at the scene, previous evidence of alcohol-related accidents or crime), medical and social records (repeated admissions due to alcohol-related problems), and forensic toxicological analysis, which were carried

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out according to the needs of cause-of-death investigations. The assessments also included interviews of family members or important collaborators. In this material, 38 cases (33 men, 5 women) had a documented history of excessive alcohol use while the control population (n = 22; 15 men, 7 women) consisted of individuals with no such records.

At autopsy, stature and weight of the body and the liver were recorded, and samples of liver tissue were processed for histological examinations. Samples of vitreous humor were collected before autopsy using a scleral puncture made on the lateral canthus of each eye. The vitreous humor was gently aspirated using a sterile syringe and needle and collected in sterile tubes that were stored at −70°C prior to analysis. Cerebrospinal fluid was aspirated with a sterile syringe from cerebral ventricles immediately after the brain was removed from the skull. Blood samples were taken in single use tubes from the femoral vein after cutting the vessel and urine samples were obtained from the bladder through dissection. In the processing of the samples, specifically designed forensic test kits and tubes containing fluoride/oxalate preservative were used. The BAC was measured by head-space gas chromatography with a limit of detection of 0.01 mg/dl. Values exceeding 10 mg/dl (0.1‰) were considered BAC positive. Serum and urine samples were prepared by centrifugation (2000 g, 10 min, 4°C) and kept frozen at −70°C until analysis.

Permission for the present study was granted by the National Authority for Medicolegal Affairs, as required in the law (Act on the Medical Use of Human Organs) following approval by the corresponding Ethics Committee (TEO 5212/04/046/07).

Biomarkers and measurements

The EtG concentration was measured using a recently developed method utilizing Microgenics EtG immunoassay reagents (Microgenics Corp., Fremont, CA, USA) and the Abbott Architect c8000 clinical chemistry analyzer (Abbott Laboratories, IL, USA) (Rainio et al., 2013). A previously established LC-MS/MS method for EtG and ethyl sulfate (EtS; used as qualifier) was used for validation of the EtG immunoassay (Helander et al., 2010; Rainio et al., 2013). For vitreous humor samples, a 1:2 dilution with 0.9% NaCl was used to improve the detection of sample surface by the analyzer (radiofrequency and pressure change detection). The measuring range of the EtG immunoassay is 0.1–2.0 mg/l and all samples showing concentrations above 2.0 mg/l were diluted with 0.9% NaCl, as required. The inter-assay coefficient of variation (%CV) for the EtG assay was 3–5% (test levels 0.4 and 0.6 mg/l). The EtG immunoassay was also found suitable for vitreous humor samples, based on good linearity over the full measuring range and excellent reproducibility of the results (intra-assay %CV of 1.7% at 0.5 mg/l EtG).

CDT levels were measured as a percentage of total transferrin (%CDT) using an automated nephelometric technique on Siemens BN Prospec analyzer and N Latex CDT immunoassay reagents (Siemens Healthcare, Erlangen, Germany). This procedure is based on a monoclonal antibody that recognizes a structural change of transferrin missing one or both N-glycans (corresponding to asialo-, monosialo- and disialotransferrin) and a highly sensitive particle-enhanced technology. To improve assay sensitivity, samples were measured without predilution and five extra calibration points were added in the low concentration range for both CDT and total transferrin assays. This approach yielded detection limits of 0.68 mg/l for CDT and 0.008 g/l for total transferrin. The observed inter-assay variations were 3–6% for CDT (test levels 4.0, 60 and 100 mg/l) and 3–5% for transferrin (test levels 0.15, 1.28 and 3.65 g/l). In the analyses of vitreous humor, isotonic NaCl solution (1:1 ratio) was added if necessary for improving the detection of sample surface by the analyzer (impedance change detection).

Statistical methods

The data are expressed as means ± SD. Differences between groups were analyzed using Student’s t-test, or non-parametric Mann–Whitney U-test for variables not showing normal distributions. Correlations between variables were calculated using the Pearson product-moment correlation coefficients or the Spearman’s rank correlations for non-continuous or skewed variables, as required. The optimal cut-offs to discriminate the alcohol-positive and alcohol-negative cases were determined from ROC (receiver operating curve) analyses maximizing the sensitivity and specificity for each marker. The area under the ROC curve (AUC) was used as a measure of the diagnostic accuracy of the test. Statistical analyses were performed using the SPSS Statistics 20 Software (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was considered statistically significant.

RESULTS

Based on medical and police records, the present study material consisted of 38 individuals with a positive and 22 individuals with a negative history of excessive alcohol consumption. Liver histological findings in the alcohol-positive group indicated that 26/38 (68%) of the cases had hepatic steatosis and 10/38 (26%) showed liver cirrhosis. In the alcohol-negative group, there were 9/22 (40%) cases with fatty liver and no subjects with liver cirrhosis.

Among the 38 cases with a documented history of excessive alcohol use, a positive BAC (median 1.15‰, range 0–3.3‰) at the time of sampling was found in 26 cases (68%). None of the subjects in the control group showed positive blood alcohol levels.

Data on EtG and %CDT levels in the different biological samples from the study groups are summarized in Table 1. In the group with a positive history of alcohol abuse, the EtG concentrations (mean ± SD) in urine (339 ± 389 mg/l, P < 0.001), vitreous humor (4.2 ± 4.8 mg/l, P < 0.001), serum (6.9 ± 8.9 mg/l, P < 0.001) and cerebrospinal fluid (1.7 ± 2.7 mg/l, P < 0.01) were all significantly higher than in the controls without such history. For %CDT, a significant difference was found only in cerebrospinal fluid (4.3 ± 2.1 vs. 2.3 ± 0.6%, P < 0.05) (Table 1).

Table 1 also summarizes the analytical characteristics of EtG and %CDT in the different samples. Biological characteristics of the sample materials hampered assays of EtG in serum and cerebrospinal fluid (i.e. hemolysis) and of %CDT in vitreous humor (impaired sample surface detection), such that a significant percentage of the specimens were unusable for analyses (Table 1). Elevated EtG concentrations in urine and vitreous humor were found in 92% of the cases with a positive history of alcohol use. Using the data obtained from medical and police records as reference, the specificity of
EtG and CDT in detecting alcohol use

Table 1. EtG and CDT concentrations in different body fluids and their diagnostic characteristics in the detection of ante-mortem alcohol use

<table>
<thead>
<tr>
<th>Alcohol-history</th>
<th>Alcohol-history</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Cut-off</th>
<th>AUC</th>
<th>Usable samples (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH-EtG (mg/l)</td>
<td>0.2 ± 0.3</td>
<td>4.2 ± 4.8***</td>
<td>92</td>
<td>91</td>
<td>0.3</td>
<td>0.94</td>
</tr>
<tr>
<td>U-EtG (mg/l)</td>
<td>10 ± 29</td>
<td>339 ± 389***</td>
<td>92</td>
<td>86</td>
<td>10</td>
<td>0.94</td>
</tr>
<tr>
<td>S-EtG (mg/l)</td>
<td>0.4 ± 0.9</td>
<td>6.9 ± 8.9***</td>
<td>84</td>
<td>88</td>
<td>0.5</td>
<td>0.89</td>
</tr>
<tr>
<td>CSF-EtG (mg/l)</td>
<td>0.1 ± 0.1</td>
<td>1.7 ± 2.7**</td>
<td>74</td>
<td>91</td>
<td>0.3</td>
<td>0.82</td>
</tr>
<tr>
<td>S-CDT (%)</td>
<td>2.0 ± 2.9</td>
<td>3.2 ± 1.8</td>
<td>72</td>
<td>71</td>
<td>2.4</td>
<td>0.69</td>
</tr>
<tr>
<td>CSF-CDT (%)</td>
<td>2.3 ± 0.6</td>
<td>4.3 ± 2.1*</td>
<td>64</td>
<td>100</td>
<td>3.4</td>
<td>0.85</td>
</tr>
<tr>
<td>VH-CDT (%)</td>
<td>1.1 ± 0.3</td>
<td>2.6 ± 2.1</td>
<td>74</td>
<td>91</td>
<td>1.3</td>
<td>0.84</td>
</tr>
<tr>
<td>BAC (‰)</td>
<td>0 (±0.1)</td>
<td>1.2 ± 1.1***</td>
<td>68</td>
<td>100</td>
<td>0.1</td>
<td>0.88</td>
</tr>
</tbody>
</table>

The data are expressed as means ± SD. **P < 0.01, ***P < 0.001 in comparison with the corresponding alcohol-negative group. Classification of cases as alcohol positive or negative was based on information from medical and police records. In urine samples, CDT could not be measured due to the lack of transferrin protein.

Table 2. Correlations between different alcohol biomarkers in post-mortem analysis

<table>
<thead>
<tr>
<th>BAC</th>
<th>VH-EtG</th>
<th>U-EtG</th>
<th>S-EtG</th>
<th>CSF-EtG</th>
<th>S-%CDT</th>
<th>CSF-%CDT</th>
<th>VH-%CDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.62***</td>
<td>0.55***</td>
<td>0.61**</td>
<td>0.53**</td>
<td>0.28</td>
<td>0.05</td>
<td>-0.07</td>
</tr>
<tr>
<td>VH-EtG</td>
<td>1</td>
<td>0.81***</td>
<td>0.81***</td>
<td>0.85***</td>
<td>0.32*</td>
<td>0.47</td>
<td>0.01</td>
</tr>
<tr>
<td>U-EtG</td>
<td>0.55***</td>
<td>1</td>
<td>0.90***</td>
<td>0.83***</td>
<td>0.53**</td>
<td>nd</td>
<td>0.14</td>
</tr>
<tr>
<td>S-EtG</td>
<td>0.61**</td>
<td>0.81***</td>
<td>1</td>
<td>0.85***</td>
<td>0.85***</td>
<td>0.27</td>
<td>nd</td>
</tr>
<tr>
<td>CSF-EtG</td>
<td>0.53**</td>
<td>0.85***</td>
<td>0.32*</td>
<td>1</td>
<td>0.37</td>
<td>nd</td>
<td>0.06</td>
</tr>
<tr>
<td>S-%CDT</td>
<td>0.28</td>
<td>0.47</td>
<td>0.58*</td>
<td>nd</td>
<td>0.88*</td>
<td>0.75</td>
<td>-0.14</td>
</tr>
<tr>
<td>CSF-%CDT</td>
<td>0.05</td>
<td>0.47</td>
<td>nd</td>
<td>0.56</td>
<td>0.75</td>
<td>0.32</td>
<td>-0.05</td>
</tr>
<tr>
<td>VH-%CDT</td>
<td>-0.07</td>
<td>-0.30*</td>
<td>-0.14</td>
<td>-0.05</td>
<td>-0.06</td>
<td>-0.32</td>
<td>-0.05</td>
</tr>
<tr>
<td>PMI days</td>
<td>-0.07</td>
<td>0.01</td>
<td>-0.14</td>
<td>-0.05</td>
<td>-0.06</td>
<td>-0.32</td>
<td>-0.05</td>
</tr>
</tbody>
</table>

BAC, blood alcohol concentration; PMI, post-mortem interval; VH, vitreous humor; U, urine; S, serum; CSF, cerebrospinal fluid; EtG, ethyl glucuronide; CDT, carbohydrate-deficient transferrin; BAC, blood alcohol concentration; AUC, area under curve from receiver operating characteristic (ROC) statistics.

DISCUSSION

The present data comparing different biomarkers of alcohol consumption in a wide variety of forensic autopsy samples indicated that EtG analyses in urine or vitreous humor show the highest diagnostic sensitivities and specificities for screening ante-mortem alcohol use. The autopsy cases were classified as alcohol-history positive or alcohol-history negative based on detailed medical (hospital case notes, toxicological analyses) and police records and investigations at the scene, which despite of their limitations have been regarded as rather reliable sources of information in forensic studies (Sadler et al., 1995).

Previously, EtG levels have been analyzed by liquid chromatography-mass spectrometric (LC-MS) techniques (Keten et al., 2009; Thierauf et al., 2011). In this work, we used a recently developed immunological assay (Rainio et al., 2013), which allows screening of different biological samples in a convenient and cost-efficient manner. This immunoassay had been validated by comparison with LS-MS/MS, a widely accepted gold standard for EtG analysis (Böttcher et al., 2008; Helander et al., 2010; Rainio et al., 2013). Current EtG measurements were found to yield markedly higher sensitivities in detecting ante-mortem alcohol use than BAC, CDT or liver histological findings. An additional possible benefit of EtG assays is also its relatively low analytical cost (typically 15–20 euros/sample) when compared with BAC assays with a typical current cost of 35–40 euros/sample.

In the present series, we found three cases with a positive history of alcohol abuse who showed EtG concentrations below the decision limit and also a negative BAC, indicating either that they had not consumed significant amounts of alcohol in the days prior to death or that the ethanol and its conjugated metabolite had disappeared prior to sampling.
(Helander et al., 2007, 2009b). While the EtG analyses were also found to yield the highest specificities, two cases classified as alcohol-history negative showed elevated EtG levels in both urine and vitreous humor specimens. While a possibility exists that impaired kidney function could result in delayed urinary excretion of EtG (Høiseth et al., 2009), recent alcohol consumption, which had escaped detection, is probably a more likely explanation for these findings since EtG was detected in two separate body fluids. In another case, serum % CDT, being a rather specific long-term biomarker of alcohol abuse, also tested positive.

In post-mortem analyses, the stability of the analyte is a key issue for the reliability of the test results (Helander et al., 2009a). Blood and urine specimens are both known to be highly susceptible to putrefaction and contamination. Cerebrospinal fluid may also become contaminated with blood upon disruption of the blood–brain barrier. Furthermore, ethanol may be formed post-mortem by micro-organisms leading to false-positive alcohol findings (O’Neal and Poklis, 1996; de Lima and Midio, 1999; Høiseth et al., 2007; Helander et al., 2009a). While some studies indicated that no EtG is formed under such conditions (Schloegl et al., 2006; Høiseth et al., 2007, 2008), other studies have demonstrated false-negative EtG findings in urine infected with Excherichia coli due to post-collection synthesis of EtG from ethanol (Helander et al., 2007). Vitreous humor is more isolated from other body fluids and is thus more protected from contamination (Harper, 1989; O’Neal and Poklis, 1996; De Martinis et al., 2006). Since vitreous humor is also expected to contain significantly less microbial activity than the other sample types (Harper, 1989), it should be less likely to yield false-positive findings. Although in the present study we found rather similar diagnostic accuracies for EtG testing in vitreous humor and urine, the former sample type may prove to be more advantageous based on its better stability. All vitreous humor samples were devoid of hemolysis, which prevented assays of EtG in nearly half of the serum and cerebrospinal fluid samples. The urine samples were also essentially free of hemolysis, but they are probably more susceptible to the action of micro-organisms (Helander et al., 2007; Ringmets et al., 2012). It should be noted, however, that at this time the kinetics of EtG formation and disappearance in different body fluids, as well as the rates of post-mortem EtG degradation, are still largely unknown (Høiseth et al., 2008, 2009, 2010; Helander et al., 2009a).

In the present material, CDT showed significant differences between the alcohol-positive and alcohol-negative groups only in assays from cerebrospinal fluid. This finding may be explained by the lack of sensitivity of CDT in detecting alcohol use (Lesch et al., 1996; Schellenberg et al., 2005; Hietala et al., 2006) but also by analytical reasons. Previous studies have reported that red cell destruction and the presence of hemolysis could interfere with the measurement of CDT (Simonnet et al., 1999; Ringmets et al., 2012). In the present study, the absolute CDT levels were, however, found not to be affected at hemoglobin concentrations <10 g/l (data not shown). There may also be an effect of the PMI on CDT results, which may be explained by transferrin degradation (Berkowicz et al., 2003). In agreement with this view, we found a significant negative correlation between PMI and serum total transferrin. In contrast, CDT and total transferrin in vitreous humor both correlated positively with the PMI, suggesting post-mortem diffusion of transferrin from blood into vitreous humor (Berkowicz et al., 2003) and supporting the view that CDT analysis in vitreous humor should perhaps only be performed if the PMI is <72 h (Berkowicz et al., 2001, 2003). As alcoholics frequently have a long PMI, this could, however, generate practical obstacles in many cases.

Taken together, the present findings support the use of EtG measurements for detecting ante-mortem alcohol consumption in cause-of-death investigations. Vitreous humor and urine appear to be the most suitable specimens for such analyses. However, since urine samples are more likely to show analytical problems, vitreous humor could be recommended as the primary specimen for forensic purposes.

Conflict of interest statement. None declared.

REFERENCES


