SCREENING AND IDENTIFICATION

Monitoring of the Alcohol Biomarkers PEth, CDT and EtG/EtS in an Outpatient Treatment Setting
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Abstract — Aims: To compare the sensitivity of whole blood phosphatidylethanol (PEth) with serum carbohydrate-deficient transferrin (CDT) as biomarkers of current regular alcohol consumption, during outpatient treatment for alcohol-related problems. Urinary ethyl glucuronide (EtG) and ethyl sulfate (EtS), and clinical assessment, were used as complementary estimates of relapse to drinking. Methods: Biomarker results for 29 men and 11 women (aged 20–73 years) undergoing voluntary outpatient treatment for harmful alcohol use or dependence were utilized for this evaluation. In connection with visits to the unit, blood and/or urine were sampled for measurement of PEth, EtG and EtS (by liquid chromatography-mass spectrometry), and CDT (%disialotransferrin, by high-pressure liquid chromatography). Results: The comparison included 326 whole blood, 319 serum (1–82 samples/patient) and 654 urine samples (1–178 samples/patient) collected over ~2 years. At the initial assessment, the total PEth value ranged between 0 and 16.5 µmol/l (mean 2.6) with 70% being above the quantification limit (0.1 µmol/l) and 55% above the reference interval (0.7 µmol/l). Initial CDT values were 0.87–6.9% (mean 2.1) with 35% above the applied reference interval (1.7%). At the final sampling (treatment period up to 21 months), the total PEth value had decreased to 0.5–9 µmol/l (mean 0.6; P = 0.0004) and CDT to 0.87–3.3% (mean 1.3; P = 0.0030). Relapses were detected by PEth alone (43% of cases), by PEth and CDT (38%) and the remainder by EtG/EtS. Conclusion: PEth was the most sensitive biomarker of current regular alcohol consumption, PEth-16:0/18:1, usually being the major subform, as was sensitive as total PEth. PEth, CDT and EtG/EtS are useful complementary tools for objective identification of current drinking and relapse detection.

INTRODUCTION

The difficulty in obtaining reliable information about the quantity and frequency of alcohol consumption and identifying early persons with hazardous alcohol use such as regular heavy or binge drinking, hamper preventive interventions for alcohol-related harm. Self-reports (e.g. clinical or telephone interview, and paper-and-pencil or computerized questionnaires) are often the primary or sole source of clinical and research data on drinking behavior (Maisto and Saitz, 2003), despite well-known problems with denial and underreporting (Helander et al., 1999). This is likely to result in numerous cases of undetected and untreated alcohol abuse and dependence, and less reliable measurement of outcomes of research studies.

Various biological measures are used to estimate alcohol consumption and detect excessive drinking in a more objective way. The analysis of alcohol (ethanol) in the breath provides a non-invasive and widely used method of approximating the blood-alcohol concentration, such as when a person is suspected of drunk driving. A major limitation of this test is the short detection window (typically <12 h) (Helander et al., 1996), due to rapid ethanol elimination. Elevated levels of ‘liver enzymes’ (i.e. GGT, ALT and AST) in blood are commonly used in clinical practice as an indicator of alcohol-induced liver damage (Niemelä and Alatalo, 2010). However, these tests suffer from low sensitivity for early detection of risky drinking, and the specificity is only moderate because many cases of elevated levels are unrelated to alcohol consumption.

Faced with these difficulties, more sensitive and specific alcohol biomarkers have been developed and demonstrated to be useful in a number of clinical and forensic settings (Erim et al., 2007; Neumann et al., 2008; Palmer, 2009; Hermansson et al., 2011), such as the detection of relapse and confirmation of abstinence during outpatient treatment. Carbohydrate-deficient transferrin (CDT) in serum (Stibler, 1991; Jeppsson et al., 2007) and phosphatidylethanol (PEth) in whole blood (Gustavsson, 1995; Isaksson et al., 2011) are biomarkers targeting regular moderate/heavy drinking before the onset of related liver damage. CDT, the first alcohol-specific biomarker in routine use, considerably reduced the risk of false identifications of alcohol-related problems, compared with using conventional liver enzymes. Later on, the introduction of sensitive and specific urinary biomarkers targeting recent drinking, i.e. the serotonin metabolite 5-hydroxytryptophol (Beck and Helander, 2003) and the conjugated ethanol metabolites ethyl glucuronide (EtG) (Schmitt et al., 1995) and ethyl sulfate (EtS) (Helander and Beck, 2004), made it possible to detect a single alcohol intake several hours up to some days afterwards, the time window largely being dose-dependent (Dahl et al., 2002; Hoiseth et al., 2008; Helander et al., 2009).

The development of an improved analytical principle for PEth based on liquid chromatography-mass spectrometry (LC-MS) (Helander and Zheng, 2009) has allowed for a more wide-spread clinical use of this alcohol biomarker. Compared with the original high-pressure liquid chromatography (HPLC) method (Aradottir and Olsson, 2005), the higher sensitivity of LC-MS methods for PEth allowed for the detection of even low/moderate drinking levels (Zheng et al., 2011). Hence, as for EtG (Helander et al., 2009, 2010), this made it necessary to establish a decision limit for PEth, to aid in test interpretation by distinguishing low/moderate (‘social’) from heavy (‘excessive’) drinking.

The present work was undertaken to compare the performance and clinical value of whole blood PEth with serum CDT measurement as biomarkers for regular excessive alcohol consumption and detection of relapse, during routine outpatient treatment of subjects diagnosed with...
alcohol-related problems. Urinary EtG and EtS measurement was employed as a complementary test for recent drinking.

MATERIALS AND METHODS

Patients and samples
The biomarker comparison was based on data from 40 outpatients (29 men and 11 women; aged 20–73 years, mean 46, median 47) diagnosed (ICD-10) with either harmful alcohol use or alcohol dependence, undergoing voluntary treatment at a small local unit for alcohol and drug dependence (1 medical doctor and 3 nurses) within the Center for Dependency Disorders (Stockholm County Council, Stockholm, Sweden). Both patients already in treatment and patients entering treatment following a recent history of heavy drinking were included. Half of the patients (20 of 40) were treated with disulfiram (Antabuse) during all or part of the study time.

The data used for this biomarker comparison were collected over a 2–year period. During return visits to the unit, blood and/or urine were sampled for alcohol biomarker measurements, and patients also received feedback on their previous test results. In addition, clinical information about treatment outcome and on the incidence of relapse drinking was collected. Retrospective information was also obtained from the medical records.

Samples of EDTA whole blood, serum and urine were kept at 4°C until transported (typically on the same day) to the Alcohol Laboratory at the Karolinska University Hospital (Stockholm). PEth, CDT and EtG/EtS are stable on storage in refrigerated samples, and this procedure also minimizes the risk for false-positive results (Stephanson et al., 2002; Helander et al., 2003; Helander and Beck, 2005; Helander and Zheng, 2009). For the scientific evaluation, all clinical and laboratory data were anonimized and only linked to the individual by a code. The study was conducted in accordance with the Declaration of Helsinki and the procedures were approved by the ethics committee at the Karolinska University Hospital.

PEth measurement in whole blood
In the laboratory, the whole blood samples were stored at −80°C until analyzed for nine PEth species by an LC-MS electrospray ionization (ESI) method run on an Agilent 1100 series LC-MS instrument (Helander and Zheng, 2009; Zheng et al., 2011). Gradient chromatography separation of a blood lipid extract was achieved on a 5-µm HyPurity C4 column (Thermo Scientific, Waltham, MA, USA) and MS analysis was performed by selected ion monitoring (SIM) in negative mode of the deprotonated molecules.

The routinely applied quantification limit for ‘total PEth’ (i.e. sum of nine PEth species) is 0.10 µmol/l. The upper limit of the 95% reference interval (URL), based on analysis of blood donor specimens, was suggested to be <0.70 µmol/l for total PEth, and <0.20 µmol/l for PEth-16:0/18:1, which is usually the predominant subform (Zheng et al., 2011).

CDT measurement in serum
The serum samples were stored at −20°C until analyzed for CDT (%disialotransferrin) using an HPLC method on an Agilent 1100 LC system (Helander et al., 2003). The method was proposed as a candidate reference method by an international federation of clinical chemistry and laboratory medicine working group of CDT standardization (Jeppsson et al., 2007). Pre-treatment of the serum sample includes iron-saturation with ferric nitrolotrpic acid and lipoprotein precipitation, followed by chromatographic separation of transferrin glycoforms on an anion-exchange column (SOURCE 15 Q, GE Healthcare, Uppsala, Sweden) by salt gradient elution. Quantification of individual glycoforms is done by monitoring the absorbance of the transferrin-iron complex at 470 nm. CDT is expressed as the percentage of disialotransferrin to total transferrin (Jeppsson et al., 2007), based on HPLC peak areas using the baseline integration mode (Helander et al., 2003).

In this outpatient study, the cut-off applied for CDT (disialotransferrin) was 1.7%, corresponding to the mean +2 SD for control populations. However, in Sweden, 1.9% disialotransferrin (i.e. mean +3 SD) is the commonly applied URL (Helander et al., 2003; Bergström and Helander, 2008a,b); it was originally chosen to ensure an extra high diagnostic specificity in medico-legal (e.g. traffic medicine) cases.

EtG and EtS measurement in urine
The urine samples were stored at −20°C until analyzed for EtG and EtS, using a routine ESI LC-MS method with SIM in negative ion mode (Helander and Beck, 2005). The ions monitored were m/z 221 for EtG, m/z 226 for EtG-d5 (penta-deuterated internal standard), m/z 125 for EtS and m/z 130 for EtS-d5 (internal standard). The EtG and EtS concentrations were calculated from the peak-area ratio to the corresponding internal standard, by reference to calibrations curves.

The routinely applied quantification limits are 0.5 mg/l for EtG and 0.1 mg/l for EtS (Helander et al., 2009, 2010).

RESULTS

The PEth and CDT biomarker comparison was based on 326 whole blood and 319 serum samples collected from 40 outpatients (range 1–82 samples/patient, mean 8.4, median 3.0). From 26 patients, 3 or more blood samples were obtained, while 12 patients only provided 1 sample each. Furthermore, from 24 of the patients, a total of 654 urine samples (range 1–178 samples/patient, mean 27, median 15) for measurement of EtG and EtS were obtained. The individual sampling time ranged up to 21 months (mean 4.8, median 2.0).

At the start of the individual sampling period, ~60–70% of patients tested positive for PEth and/or CDT, the range depending on the cut-offs compared (Table 1). During treatment, the frequency of patients showing at least one positive biomarker value was ~50–60%. Of the cases who had at least one positive test result (PEth or CDT), about half had both positive, and about half only PEth positive. In only one case, CDT was the single positive biomarker (Table 1).

The total PEth value at the initial assessment ranged between 0 and 16.5 µmol/l (mean 2.6, median 1.5) with 70% of samples containing >0.1 µmol/l (quantification limit), 60% >0.5 µmol/l and 55% >0.7 µmol/l (URL) (Fig. 1). At the end of the individual sampling period, the total PEth values had dropped significantly to 0–5.9 µmol/l (mean 0.58, median 0.00; P = 0.0004, Wilcoxon test for paired samples).
Immediately after the delivery, her values returned to normal.

There was an overall good correlation ($r = 0.62$) between the PEth and CDT values for the entire data set comprising 303 pairs from 40 patients, albeit with a large scatter (Fig. 3a). The intra-individual correlation between PEth and CDT values was considerably better ($r$ typically $>0.8$), although the relative responses were highly individual as reflected in markedly different slopes and intercepts for the regression lines (Fig. 3b).

The patients were classified as either ‘sober’ or ‘relapsing’, based on the alcohol biomarker results during the sampling period. Patients classified as relapsing ($n = 21$), defined as showing at least one positive biomarker value, were detected by PEth alone in nine cases (43%) (PEth being formed only in the presence of ethanol and thus a very specific alcohol biomarker), and by both PEth and CDT in eight (38%) cases. Seven of the PEth and/or CDT-positive patients also tested positive for urinary EtG/EtS at least once. Another four patients tested positive only for EtG/EtS, indicating recent occasional, but not regular, drinking.

It should be noted that urine samples were not obtained from 16 of the 40 patients. Among 11 patients who did not provide urine samples for EtG/EtS testing but gave at least three blood samples for measurement of PEth and CDT, there was an apparent difference in the distribution between patients classified as sober and relapsing (Fig. 4). However, due to the small number of observations, this difference did not reach statistical significance ($P = 0.136$).

Patients showing a steady decline in biomarker values after the initial assessment or following a relapse into heavy drinking during the study, but without indications of further drinking according to urinary EtG/EtS, were used to estimate the half-lives for PEth and CDT (i.e. by plotting log values vs. time for 2–3 consecutive samples). The half-lives for total PEth calculated in this way ranged between 3.5 and 9.0 days (mean 6.1, median 7.0; $n = 11$) and for

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**Table 1. CDT and PEth values at the initial assessment and during outpatient treatment**

<table>
<thead>
<tr>
<th>Biomarker values</th>
<th>Test result at start of study ($n$ %)</th>
<th>Test result during treatment ($n$ %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutoffs: CDT &gt;1.7%; total PEth &gt;0.1 µmol/lc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDT and total PEth negative</td>
<td>11 (27.5%)</td>
<td>9 (34.6%)</td>
</tr>
<tr>
<td>CDT and total PEth positive</td>
<td>13 (32.5%)</td>
<td>9* (34.6%)</td>
</tr>
<tr>
<td>CDT positive, PEth negative</td>
<td>1 (2.5%)</td>
<td>0</td>
</tr>
<tr>
<td>CDT negative, PEth positive</td>
<td>15 (37.5%)</td>
<td>8 (30.8%)</td>
</tr>
<tr>
<td>Cutoffs: CDT &gt;1.7%; total PEth &gt;0.7 µmol/lc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDT and total PEth negative</td>
<td>17 (42.5%)</td>
<td>13 (50.0%)</td>
</tr>
<tr>
<td>CDT and total PEth positive</td>
<td>13 (32.5%)</td>
<td>8* (30.8%)</td>
</tr>
<tr>
<td>CDT positive, PEth negative</td>
<td>2 (5.1%)</td>
<td>1* (3.8%)</td>
</tr>
<tr>
<td>CDT negative, PEth positive</td>
<td>9 (22.5%)</td>
<td>4 (15.4%)</td>
</tr>
<tr>
<td>Cutoffs: CDT &gt;1.7%; PEth-16:0/18:1 &gt;0.2 µmol/lc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDT and PEth-16:0/18:1 negative</td>
<td>13 (32.5%)</td>
<td>12 (46.2)</td>
</tr>
<tr>
<td>CDT and PEth-16:0/18:1 positive</td>
<td>13 (32.5%)</td>
<td>8* (30.8%)</td>
</tr>
<tr>
<td>CDT positive, PEth-16:0/18:1 negative</td>
<td>2 (5.1%)</td>
<td>1* (3.8%)</td>
</tr>
<tr>
<td>CDT negative, PEth-16:0/18:1 positive</td>
<td>13 (32.5%)</td>
<td>5 (19.2%)</td>
</tr>
</tbody>
</table>

*All patients ($n = 40$).

*Only patients from which ≥3 blood samples were obtained ($n = 26$). A negative test result during treatment implies that all samples tested negative for the biomarkers.

*The cut-offs employed to indicate a positive test result was 1.70% for CDT (%disialotransferrin) in serum (Helander et al., 2003; Arndt et al., 2008b), 0.10 µmol/l (‘any regular drinking’) and 0.70 (‘excessive drinking’) for total PEth, and 0.20 (‘excessive drinking’) for PEth-16:0/18:1 in whole blood (Zheng et al., 2011), respectively.

*One of these patients consistently showed incomplete separation between disialo- and trisialotransferrin (i.e. a C2C3 genotype or di/tri-bridging pattern) (Helander et al., 2003; Arndt et al., 2008) that prevented reliable quantification of the %disialotransferrin level.

*In this female patient, pregnancy was the likely cause for two borderline positive CDT samples during the third trimester (Kenan et al., 2011). Immediately after the delivery, her values returned to normal.

For CDT, the relative disialotransferrin value at the initial sampling ranged between 0.87 and 6.9% (mean 2.1, median 1.4), 35% being >1.7% (URL) and 30% >1.9% (Fig. 1). In the final sample, the values had dropped significantly to 0.23 µmol/l (mean 0.22, median 0.00; $P < 0.0001$) at the end (Fig. 2b).

For CDT, the relative disialotransferrin value at the initial assessment among 40 outpatients exceeding different threshold limits at the initial assessment among 40 outpatients. For total PEth, 0.1 µmol/l is the routinely applied quantification limit and 0.7 µmol/l the upper limit of the 95% reference interval (URL) based on results from blood donors. The URL for PEth-16:0/18:1 is 0.2 µmol/l. For CDT, 1.7% disialotransferrin corresponds to the mean + 2 SD and 1.9% to the mean + 3 SD for control populations.
%disialotransferrin between 8.5 and 15 days (mean 12.6, median 13.9; n = 6).

Besides the alcohol biomarker results, patient self-reports of drinking were routinely collected during visits to the unit, and additional retrospective data were retrieved from the medical records after the study was completed. Among patients classified as sober based on showing only negative biomarker values, there were no self-reports of drinking or indications of undetected drinking. Among patients with one or more positive biomarkers during follow-up, almost all admitted some alcohol intake (detailed frequency data were not available), but obviously not on every occasion, and/or the true amount may not have been given, and some only admitted drinking some time afterwards. However, a few patients admitted drinking only after being confronted with a positive biomarker value.

The retrospective information retrieved from medical records further indicated a complex picture with different clinical outcomes for this group of patients, from apparently complete sobriety, or return to social drinking and no further contact with this or other treatment units (classified as ‘positive outcomes’), to continued treatment for alcohol-related problems, need for Antabuse treatment, frequent relapses (classified as ‘negative outcomes’), and even deaths. The frequency of positive outcomes was higher among patients treated with Antabuse (55 vs. 10%), and the frequency of negative outcomes was higher among patients not taking Antabuse (70 vs. 30%). As for the five (12.5%) patients who died, all were classified based on biomarkers as relapsing and three had been treated with Antabuse and two not.

DISCUSSION

The results of this alcohol biomarker comparison study indicated that the outpatient treatment program was significantly related to reduced overall alcohol consumption, as reflected in reduced alcohol biomarker levels during the course of the sampling period. In some patients, beginning frequent testing with sensitive alcohol biomarkers combined with individual feedback on test results appeared to be helpful in reaching abstinence following a period of hidden heavy drinking. Nevertheless, retrospective data from the medical records also revealed that the final outcome for many patients was
were given urine samples for EtG/EtS testing of recent drinking and (result) during treatment. Data are presented separately for (moderate drinking) Zheng [citation limit will not only detect heavy but also regular low/difference is that the highly sensitive LC-MS methodology of current regular alcohol consumption, and was found biomarker, demonstrated the highest sensitivity for the detection in the presence of ethanol and is thus a very specific alcohol being high. far from positive, the frequency of relapse and even death is being high.

Of the long-term biomarkers, PEth, which is formed only in the presence of ethanol and is thus a very specific alcohol biomarker, demonstrated the highest sensitivity for the detection of current regular alcohol consumption, and was found positive about twice as often as CDT. One reason for this difference is that the highly sensitive LC-MS methodology used for PEth measurement combined with a low quantification limit will not only detect heavy but also regular low/moderate drinking (Zheng et al., 2011), and apparently even a single large alcohol intake. In one patient, drinking 85 g ethanol on one occasion resulted in a total PEth value of 0.3 µmol/l five days later. Nonetheless, in about 60% of PEth positive cases, the total value exceeded 0.7 µmol/l (95% reference interval based on 200 random samples from blood donors), which was recently suggested as a threshold to indicate excessive drinking (Zheng et al., 2011), but the consumption was apparently not high and/or frequent enough for CDT to become elevated. Overall, however, there was a good correlation between the PEth and CDT values, although the large scatter indicated a substantial inter-individual variability in biomarker response. This was further supported by the observation of a much better intra-individual agreement between PEth and CDT, but with highly different relative responses.

The present results further indicated that measurement of PEth-16:0/18:1, which is typically the major PEth subform (Helander and Zheng, 2009; Zheng et al., 2011), did not markedly change the sensitivity of PEth testing. Being able to focus the LC-MS analysis on a single subform, instead of a total amount, would represent an obvious advantage and facilitate the standardization of PEth analysis.

In one patient, the CDT level at the initial assessment was markedly elevated, although no PEth was detected. This might be explained by the shorter half-life of PEth, as also indicated by the present results, because this subject had elevated values of both CDT and PEth at a subsequent sampling in connection with a relapse after the study was completed. The estimated half-life for PEth in whole blood (median ~7 days) was somewhat longer than the ~4 days previously reported (Varga et al., 2000). Actually, an even shorter half-life was indicated in a recent detoxification study (Wurst et al., 2010). Controlled studies are required to determine inter-individual variations in PEth turnover and responses to different levels and times of drinking.

Besides PEth and CDT, several drinking occasions were only detected through the analysis of the urinary ethanol metabolites EtG and EtS. This was expected, given that these highly sensitive indicators of recent drinking can detect even a single small/moderate intake of alcohol. Although the urinary excretion of EtG and EtS accounts for <0.1% of the ingested ethanol dose, both remain detectable for considerably longer than ethanol itself, the time-lag largely being dose dependent (Dahl et al., 2002; Hoiseth et al., 2008; Helander et al., 2009). Because EtG and EtS are direct ethanol metabolites, a positive finding is regarded as a very reliable indicator of recent drinking, even in cases when ethanol is not detected. In the present comparison of PEth and CDT, the risk of relapse was lower among patients who also gave urine samples for measurement of EtG/EtS. This observation, although not reaching statistical significance, might be related to the frequency of return visits at the unit for sampling and receiving feedback on test results.

In conclusion, given the well-known limitations of self-report data, the results of this outpatient treatment study highlighted the clinical value of alcohol biomarkers as objective measures of current and prior drinking. PEth measurement in whole blood was a more sensitive biomarker than serum CDT for the detection of relapse drinking, because the PEth test can detect lower consumption levels. However, the considerable inter-individual variability in PEth levels observed in clinical studies (Aradottir et al., 2006; Stewart et al., 2009, 2010; Naalesso et al., 2011), and also demonstrated here, may create problems in the interpretation of results, if the test is applied to indicate risky drinking in connection with health and lifestyle examinations (Hermansson et al., 2011). This suggests that a PEth value in the low concentration range is useful mainly to differentiate ‘any drinking’ (e.g. relapse) from abstinence during outpatient treatment for alcohol-related problems, rather than to indicate with confidence a specific amount of consumption. The results of this study further pointed to the value of combining biomarkers of short-term and long-term drinking. CDT focuses on current heavy consumption (Conigrave et al., 2002), whereas urinary EtG/EtS is useful to detect or rule out recent intake of even small amounts of alcohol. Based on the favourable outcome of this clinical biomarker comparison, the outpatient unit is running a 1-year treatment program for drink-drive offenders, which offers the option of demonstrating abstinence from drinking as an alternative to an ankle monitor sentence or prison.

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REFERENCES


