

Phosphatidylethanol: The Potential Role in Further Evaluating Low Positive Urinary Ethyl Glucuronide and Ethyl Sulfate Results

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Background: Whereas urinary ethyl glucuronide (EtG) levels above 1,000 ng/ml reflect with a high probability ethanol (EtOH) consumption, levels below this cutoff are difficult to interpret as both extraneous (nonbeverage) EtOH exposure, recent drinking, and more distant high EtOH intake (several days ago) might yield similar results. This might be of particular relevance in medico-legal cases. To overcome this dilemma, phosphatidylethanol (PEth) might be a promising marker, because blood PEth is only positive following significant alcohol use. The aim of our study was therefore to employ PEth as a marker to differentiate between the different conditions.

Methods: Subjects included were 252 participants in monitoring with the Alabama Physician Health Program. All subjects testing positive for EtG and/or ethyl sulfate (EtS) who denied drinking after routine supportive confrontation were subject to information about PEth testing. If they still denied drinking, PEth testing was performed and the result communicated. EtG, EtS, and PEth testing was performed in a commercial laboratory using liquid chromatography tandem mass spectrometry methods.

Results: Of a total of 18 subjects who tested positive for EtG and/or EtS, 10 denied drinking. Of the 7 who denied drinking after PEth explanation, in 5 cases, their claim was supported by a negative PEth result. In 2 cases, a positive PEth result was in contrast to their claim.

Conclusions: PEth results in combination with previous low positive EtG/EtS results allow differentiating between innocent/extraneous exposure and drinking. Negative PEth testing following low positive EtG/EtS results helps to further elucidate the findings and support the claim of the patient of recent alcohol abstinence. Positive PEth testing following positive EtG/EtS results confirms recent drinking.

Key Words: Ethyl Glucuronide, Ethyl Sulfate, Phosphatidylethanol, Physician Health Program, Monitoring.

ETHYL GLUCURONIDE (ETG) and ethyl sulfate (EtS) are direct ethanol (EtOH) metabolites gaining increased usage over the past decade as biomarkers of EtOH intake in professional monitoring programs, courts, schools, transplant clinics, and others (for review, see Walsham and Sherwood, 2012). They are advantageous as compared with traditional biological state markers, such as liver enzymes or mean corpuscular volume, because those are indirect measures of EtOH intake and are limited with regard to the time

period for previous drinking and/or are confounded due to age, gender, other ingested substance intake, and nonalcohol-associated diseases (Allen et al., 2009; Conigrave et al., 2002; Hannuksela et al., 2007; Helander, 2003; Laposata, 1999; Walsham and Sherwood, 2012). The newer markers are therefore more effective in documenting abstinence, detecting relapse, and deterring drinking (Skipper et al., 2004; Wurst et al., 2003).

Soon after urine EtG/EtS testing began, claims of false accusations of drinking from positive tests emerged associated with numerous reports of positive tests from extraneous alcohol in various products (Hoiseth et al., 2010; Rohrig et al., 2006). The list of "nonbeverage" products containing ethyl alcohol is extremely long and includes mouthwashes, many over-the-counter medications, "alcohol-free" wine and beer, communion wine, foods and desserts cooked with alcohol, and many others. Even items such as ripe fruits, fruit juices, sauerkraut, and even soft drinks contain alcohol due to fermentation and have been shown to cause positive EtG/EtS tests (Mushoff et al., 2010). Following the discovery that hand-sanitizing gels cause positive EtG/EtS tests (Rohrig et al., 2006), the list of sources of incidental exposure to alcohol was further expanded to include topical products containing alcohol such as insecticides, cleaning products,

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topical medications containing alcohol (e.g., testosterone gel), and colognes, etc. Furthermore, a recent report documented that subjects administered brewer's yeast and sugar by mouth caused elevated EtG/EtS levels, presumably from fermentation within the gastrointestinal tract (Thierauf et al., 2010). Therefore, unlike poppy seeds that contain small amounts of opium that can cause positive tests for morphine and are relatively easy to avoid, there are hundreds of products that contain EtOH. It is troubling that so many products can cause positive tests when it is only detection of drinking of alcohol that is sought. Therefore, in particular persons in medico-legal contexts such as monitoring programs need clear and documented advice regarding potential sources of extraneous EtOH exposure to avoid difficulties in interpreting EtG results.

Complaints from health professionals and others in monitoring who claimed they had been falsely accused and punished and had not been drinking alcohol and numerous reports of extraneous EtOH exposure causing positive tests led the Substance Abuse and Mental Health Services Administration (SAMHSA, 2012) to issue an advisory in 2006 warning that a positive EtG test is not proof of drinking and should not be used to punish or sanction individuals. Further complicating the issue was a report that in some situations bacteria can both destroy and/or create EtG *in vitro* (Baranowski et al., 2008; Helander et al., 2007). The use of stabilizers, however, can effectively solve this issue. The wider use of EtS was fostered by the fact that EtS is not affected by bacterial action. Both markers have a similar time frame of detection. EtS, however, is subject to the same effects from extraneous exposure as EtG and does not solve the problem of differentiating extraneous exposure from actual alcohol use. An immunoassay is available for EtG testing and yields reasonable results for clinical settings, not however for medico-legal cases (Böttcher et al., 2008; Helander et al., 2009). No such test exists for EtS to date.

Various estimates for cutoff values that might distinguish between extraneous exposure and actual drinking have been suggested based on reports of exposure studies. Unfortunately, the available data have been based on very small numbers of exposure studies, utilizing very few healthy subjects exposed to single items. With so many hundreds of items containing alcohol, it is easily conceivable that there could be multiple exposures to 2 or more sources of alcohol, which would be additive; therefore, single-item exposure studies would not reflect the levels that could be achieved. Additionally, larger populations of subjects with various illnesses, medications, and conditions are needed, because these factors can affect enzyme systems and resulting levels of these analytes.

Therefore, there is no universally agreed upon cutoff that accurately distinguishes between drinking and extraneous exposure. However, based on the fact that exposure studies never yielded results above 1,000 ng/ml, a differential cutoff has been suggested of 1,000 ng/ml or more for EtG to confirm drinking (Thierauf et al., 2009). In addition, a recent

revision of the SAMHSA advisory suggests that values of between 500 and 1,000 ng/ml could be from previous drinking as well as from recent intense extraneous exposure (within 24 hours or less; SAMHSA, 2012).

Whereas EtG levels above 1,000 ng/ml with a high probability reflect EtOH consumption, levels below this cutoff are difficult to interpret as extraneous alcohol exposure, recent drinking and more distant EtOH intake (several days) might yield the same results. To overcome this dilemma, phosphatidylethanol (PEth) holds promise as a marker to differentiate between the different conditions as blood PEth is only positive following significant alcohol use.

PEth is an abnormal phospholipid formed in the presence of EtOH via the action of phospholipase D (Gustavsson and Alling, 1987; Kobayashi and Kanfer, 1987). Initial reports using the high-performance liquid chromatography (HPLC) method found PEth positive in alcoholics and suggested that a threshold of total EtOH intake yielding detectable PEth seems to be around 1,000 g, with a mean daily intake of about 50 g (Hansson et al., 1997; Varga et al., 1998, 2000). Once positive it remains positive for 2 to 3 weeks or more (Wurst et al., 2010, 2012). Additionally, no gender differences have been found (Wurst et al., 2012), several studies reported no false-positive results (Hartmann et al., 2007; Wurst et al., 2003, 2004, 2010), and a linear correlation between amount of EtOH consumed and PEth values seems probable (Aradottir et al., 2006; Stewart et al., 2009, 2010). Recently, blood PEth testing has become commercially available. Whereas initially PEth was measured using HPLC, in recent years liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods are used (Faller et al., 2011; Gnann et al., 2010, 2012; Helander and Zheng, 2009; Stewart et al., 2010). In 2010, the identification of 48 PEth homologues was reported (Gnann et al., 2010). The lower limit of detection (LOD) and limit of quantitation (LOQ) when using the LC-MS/MS method result in an increased sensitivity. This aspect changed the marker characteristics since in addition to information regarding chronic intake even single intoxications of above 0.1 now can be detected.

In a recent drinking experiment, 11 test persons drank an amount of EtOH on each of 5 successive days leading to an estimated blood EtOH concentration of about 1 g/kg. In 10 of 11 volunteers detectable PEth 16:0/18:1, an abundant homologue, values were found 1 hour after the start of drinking. Over the following days, concentrations of PEth increased and reached the maximum concentrations between days 3 and 6. The highest concentration found was 237 ng/ml (Gnann et al., 2012). An additional benefit to PEth testing is that blood is not subject to the confounding effects of concentration. These facts and the time spectrum of detection make blood PEth a potential confirmation test for drinking.

The aim of our study was therefore to employ PEth as a marker to differentiate between extraneous incorporation, recent drinking, and more distant high EtOH intake (several

days ago) in urine EtG/EtS positive subjects who deny drinking.

MATERIALS AND METHODS

We included 252 participants in monitoring in the Alabama Physician Health Program. Among those were 222 physicians, 7 residents and medical students, 3 veterinary technicians, and 20 veterinarians. All subjects signed informed consent for monitoring. The study was approved by the ethics committee of the Medical Association of the State of Alabama. During the course of routine drug testing, EtG/EtS testing were utilized on a random basis from between weekly (at the beginning of testing) to monthly for subjects who remained abstinent for at least 6 months.

Beginning in June 2010, with the availability of blood PEth testing, all subjects testing positive for EtG and/or EtS who denied drinking after routine supportive confrontation were subject to the following protocol:

1. Subjects were advised that a new marker is available that can accurately confirm drinking and is not affected by extraneous exposure. They were advised that this "confirmation" test would need to be performed. The sensitivity and specificity of the test was explained as well as the procedure for obtaining the test and its cost.
2. They were again supportively asked whether they were sure they had not been drinking. If they continued to deny drinking, the PEth test was obtained. The laboratory made arrangements with collection sites, sent supplies, and instructions regarding how to obtain and send the samples.
3. If the PEth test was positive (> 20 ng/ml), the subject was again confronted and then referred for further multidisciplinary evaluation (with or without admission of drinking).
4. If the PEth test was negative (< 20 ng/ml), the subject was given routine warning information regarding avoidance of extraneous exposure to alcohol and was returned to routine monitoring.

EtG, EtS, and PEth testing were performed in a commercial laboratory, United States Drug Test Lab (USDTL) in Chicago, using LC-MS/MS methods. LOQ and LOD for EtG were 90 and 36 ng/mL and for EtS 18 and 7 ng/mL, respectively (Helander and Beck, 2005; Stephanson et al., 2002). USDTL which is one of the major

laboratories reports results for EtG and EtS positive above the cutoff of 250 and 50 ng/ml, respectively.

For PEth also an LC-MS/MS method was used (Helander and Zheng, 2009). The method monitored a single isomer of PEth (palmitoyl/oleoyl), which is the most prevalent PEth species. The LOD was 8 ng/mL, the LOQ was 19 ng/mL, and the assay was linear up to 800 ng/mL. For PEth a clinical cutoff of 20 ng/ml was used.

Data for this study were obtained from actual laboratory reports from an existing ongoing professionals monitoring program utilizing the testing protocols, cutoffs, and policies in effect at that time for the program.

RESULTS

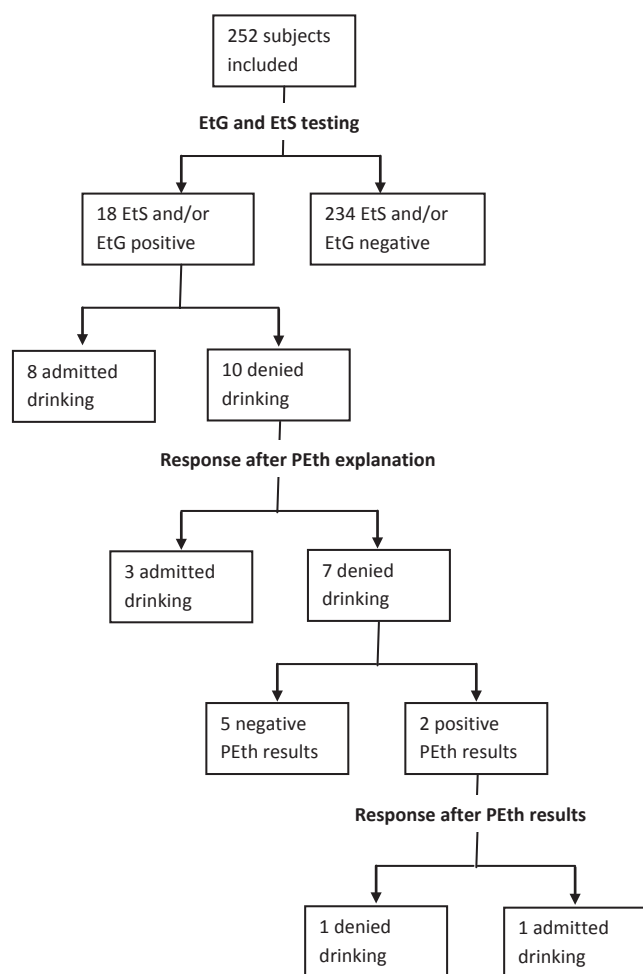
During the 8 month period between June 1, 2010 and February 28, 2011 of a total of 252 persons who received a total of 383 EtG/EtS tests, 18 subjects tested positive for EtG and/or EtS. Eight (44%) of the 18 subjects with a positive EtG, and/or EtS admitted drinking. An additional 17% ($n = 3$ of 18) admitted drinking after blood PEth testing was explained and 1 subject (6%) additionally admitted drinking after a positive PEth test. Therefore, simply adding the PEth test increased the number of admissions of drinking from 44 to 67%, a 33% increase. Additionally, 6% ($n = 1$ of 18) had a positive blood PEth after denying drinking, which was relied upon as a confirmation of drinking. This individual later admitted drinking while in treatment. Only in 1 instance, a PEth test was performed following admission of drinking. The PEth result was positive at 480 ng/ml. For further details see Table 1 and Fig. 1.

Both the medical director of the program and the counselor responsible for compliance tracking agreed that this methodology was preferable, even knowing that some individuals with small amounts of drinking may not be detected compared with the difficulties associated with the chance of false accusation of drinking without this step.

Table 1. Test Results for Ethyl Glucuronide, Ethyl Sulfate, and Phosphatidylethanol and Subjects Response

Subject	U ₁₀₀ EtG, ng/ml	U ₁₀₀ EtS, ng/ml	Response before PEth explained	Response after PEth explained	PEth result, ng/ml	Response after PEth result	Response after evaluation
1	368	121	Admitted drinking				Underwent further treatment
2	1,267	413	Admitted drinking				Underwent further treatment
3	2,034	Neg	Denied drinking	Admitted drinking			Underwent further treatment
4	516	140	Denied drinking	Denied drinking	220	Admitted drinking	Underwent further treatment
5	>7,500	>2,500	Denied drinking	Admitted drinking	480		Underwent further treatment
6	380	122	Admitted drinking				Underwent further treatment
7	224	75	Denied drinking	Denied drinking	Neg		Continued monitoring
8	>7,500	>2,500	Admitted drinking				Underwent further treatment
9	492	128	Admitted drinking				Underwent further treatment
10	339	110	Denied drinking	Denied drinking	Neg		Continued monitoring
11	222	96	Denied drinking	Admitted drinking			Underwent further treatment
12	788	256	Admitted drinking				Underwent further treatment
13	373	167	Denied drinking	Denied drinking	Neg		Continued monitoring
14	888	340	Denied drinking	Denied drinking	320	Denied drinking	Underwent further treatment
15	556	192	Admitted drinking				Underwent further treatment
16	2,246	489	Admitted drinking				Underwent further treatment
17	868	366	Denied drinking	Denied drinking	Neg		Continued monitoring
18	343	178	Denied drinking	Denied drinking	Neg		Continued monitoring

U₁₀₀EtG: urinary ethyl glucuronide normalized to creatinine 100 mg/dl, cutoff 250 ng/ml; U₁₀₀EtS: urinary ethyl sulfate normalized to creatinine 100 mg/dl; cutoff 50 ng/ml; PEth: phosphatidylethanol, cutoff 20 ng/ml.



Legend: EtG: ethyl glucuronide, EtS: ethyl sulfate, PEth: phosphatidylethanol

Fig. 1. Flow chart of the sequence of testing.

DISCUSSION

The major finding of this pilot study is that the combination of urinary EtG/EtS testing and PEth testing seems effective in providing additional information on potential recent drinking: (i) PEth results in combination with previous low positive EtG/EtS results allow differentiating between innocent/extraneous exposure versus drinking. (ii) Negative PEth testing following low positive EtG/EtS results helps to further elucidate the findings and support the claim of the patient of recent alcohol abstinence. (iii) Positive PEth testing following positive EtG/EtS results confirms recent drinking.

In this study 24% of positive EtG/EtS tests did not result in admission of drinking or confirmation of drinking. It is possible that some of these individuals did drink but were under the threshold for a positive blood PEth test. However to date, none of these individuals have had further evidence of relapse or admission of drinking.

Strengths and Limitations

To our knowledge, this is the first study to employ the marker combination in the above-mentioned sequence. The suggested algorithm seems promising in situations where differentiating innocent/extraneous exposure versus drinking is important. This might in particular be the case in medico-legal issues such as physician health program, child custody cases, or monitoring abstinence in DUI offenders. A clear limitation of this study is the limited number of positive EtG/EtS and PEth tests. Large-scale studies and long-time monitoring of the outcome are required.

A positive test result for EtG below 1,000 ng/ml even if confirmed by a positive EtS test is sensitive for any type of EtOH exposure, however not specific for drinking. Because blood PEth is only positive following significant alcohol use, far more significant than could normally be attained from extraneous exposure, it is more specific for drinking. Therefore, it seems promising to use these tests in combination: EtG/EtS to screen for possible drinking followed by blood PEth, to allow differentiating between innocent/extraneous exposure and drinking. When drawing blood samples, it is of importance to consider that elevated PEth levels have been reported due to in vitro formation of PEth in the presence of higher EtOH concentrations in blood when stored at room temperature or -20°C . In contrast, blood samples that contain EtOH can be stored refrigerated for up to 72 hours or frozen in liquid nitrogen and stored at 80°C without affecting PEth levels (Aradóttir et al., 2004).

A promising new method that has proven to be as reliable as the determination in whole blood is the determination of PEth species in dried blood spots (Faller et al., 2011). Advantages include the fact that sampling is possible by non-medical personnel since capillary blood is used, there is no venipuncture and the transport is easier, as no special containers, and no cooling is required.

To conclude, EtG and EtS can be considered to be highly sensitive in detecting alcohol intake. However, to overcome the dilemma in interpreting low positive EtG/EtS results, the use of PEth testing seems to be effective in providing additional information on potential recent drinking or extraneous EtOH exposure.

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