

Application of phosphatidylethanol (PEth) in whole blood in comparison to ethyl glucuronide in hair (hEtG) in driving aptitude assessment (DAA)

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Acknowledgement

We would like to thank the team of the Department of Traffic Sciences of the Institute of Forensic Medicine Bern for collecting the blood and hair samples for comparison of PEth in blood and EtG in hair (hEtG) in driving aptitude assessment, and we would like to thank Anja Kaiser, Nadja Utiger, Sidonia Guggisberg, Anita Iannone, Severine Krönert and Thomas Wüthrich from our institute for the determination of ethyl glucuronide in hair.

Funding

This study was supported by the Swiss Foundation of Alcohol Research (Grant 254/2014: Studies on phosphatidylethanol (PEth) – a promising biomarker for the detection of harmful ethanol consumption – and its possible use for abstinence monitoring).

Abstract

For driving aptitude assessment (DAA), the analysis of several alcohol biomarkers is essential for the detection of alcohol intake besides psycho-medical exploration.

In Switzerland, EtG in hair (hEtG) is often the only direct marker for abstinence monitoring in DAA. Therefore, the suitability of phosphatidylethanol (PEth) was investigated as additional biomarker.

PEth 16:0/18:1 and 16:0/18:2 were determined by online-SPE-LC-MS/MS in 136 blood samples of persons undergoing DAA and compared to hEtG, determined in corresponding hair segments.

With a PEth 16:0/18:1 threshold of 210 ng/mL for excessive alcohol consumption, all (n=30) but one tested person also had hEtG values ≥ 30 pg/mg. In 54 cases, results are not in contradiction to an abstinence as neither PEth (< 20 ng/mL) nor hEtG (< 7 pg/mg) was detected. In 8 cases both markers showed moderate consumption. Altogether, PEth and hEtG were in accordance in 68% of the samples, although covering different time periods of alcohol consumption.

With receiver operating characteristic analysis, PEth was evaluated to differentiate abstinence, moderate and excessive alcohol consumption in accordance with hEtG limits. A PEth 16:0/18:1 threshold of 150 ng/mL resulted in the best sensitivity (70.6%) and specificity (98.8%) for excessive consumption. Values between 20-150 ng/mL passed for moderate consumption, values < 20 ng/mL passed for abstinence.

As PEth mostly has a shorter detection window (2-4 weeks) than hEtG (up to 6 months depending on hair length), changes in drinking behavior can be detected earlier by PEth than by hEtG analysis alone. Therefore, PEth helps to improve the diagnostic information and is a valuable additional alcohol marker for DAA.

Keywords

Phosphatidylethanol (PEth) in blood, Alcohol biomarker, Ethyl glucuronide in hair (hEtG), Online-SPE-LC-MS/MS

Introduction

In Switzerland, administrative regulations require a driving aptitude assessment (DAA) for convicted driving under the influence (DUI) of alcohol offenders having a blood alcohol concentration of 1.6 ‰ and higher, as prolonged excessive alcohol misuse is suspected above this limit [1, 2]. In consequence of a DUI of alcohol offence, the driver's license is revoked. The driver has to pass a DAA, in which the driver's alcohol consumption habits are evaluated. Generally, the affected persons have to prove abstinence over a time period of 6 months (after traffic relevant alcohol misuse) up to 12 months (alcohol dependency) prior to re-granting the driver's license, and after re-granting the driver's license, minimal 12 months of a controlled alcohol abstinence are stipulated [3].

For DAA direct (ethyl glucuronide EtG, ethyl sulfate EtS, fatty acid ethyl esters FAEE) and indirect markers (carbohydrate deficient transferrin CDT, γ -glutamyl transferase GGT, mean corpuscular volume MCV, aspartate aminotransferase ASAT, alanine aminotransferase ALAT) are used [4]. However, EtG in hair (hEtG) often is the only direct marker for abstinence monitoring during DAA in Switzerland.

Hair samples mainly consist of head hair from the posterior region (0 – 5 cm) of the scalp, sometimes also from arms, legs and chest.

EtG is a non-volatile, water-soluble metabolite of ethanol (figure 1) [5]. It is formed by a non-oxidative phase II metabolism, catalyzed enzymatically by UDP-glucuronosyl transferases, which conjugates ethanol with activated glucuronic acid [6]. This reaction constitutes only a very small fraction (<0.1%) of the ingested alcohol dose [7, 8]. Depending on the amount of consumed alcohol EtG is detectable in blood, urine, tissues, sweat and hair long time after termination of ethanol elimination [5, 9].

Compared to the smaller detection windows of EtG in body fluids, EtG is detectable for several months in hair depending on the hair length. Therefore, hEtG is of advantage for long-term abstinence monitoring in DAA. To differentiate prolonged excessive from moderate alcohol consumption the Society of Hair Testing (SOHT) proposes a threshold of 30 pg/mg hEtG. A threshold of 7 pg/mg is “non-contradicting to self-reported abstinence”. This expression is used, as occasional alcohol consumption cannot always be excluded, and if samples less than 3 cm or greater than 6 cm are analyzed for EtG, the results should be interpreted with caution [8, 10-12].

A direct marker, which has also a potential in the field of abstinence monitoring, is phosphatidylethanol (PEth), as it is formed directly after alcohol intake [13, 14].

The term PEth represents a group of phospholipid homologues, which are ethyl-esters of phosphatidic acid (figure 1). These homologues have two fatty acid chains bound to the glycerol backbone in sn-1 and sn-2 position and phosphoethanol in sn-3 position. The fatty acid chains typically contain 14 – 22 carbon atoms with 0 – 6 double bonds [15, 16]. PEth homologues are present in cell membranes [17] and are biosynthesized from phosphatidylcholine (PC) catalyzed by the enzyme phospholipase D (PLD) [18], as long as ethanol is present in the organism. Due to an elimination half-life of about four days [17], accumulation of PEth is observed after repeated drinking. Therefore, PEth has been discussed as marker for detection of prolonged excessive alcohol consumption in several studies [16, 19-23].

Furthermore, drinking studies [14, 24] with a repeated alcohol consumption leading to a blood alcohol concentration (BAC) of 1 g/kg on each of five or ten subsequent days showed a correlation between degree of alcohol consumption and detected PEth concentrations. The potential of PEth in abstinence monitoring was shown in another drinking study: After a single drinking event leading to an estimated BAC of 1 g/kg and followed by an abstinence period of two weeks, PEth was detectable for three up to twelve days [25].

In this study we investigate the suitability of PEth in blood as an additional diagnostic marker for the assessment of drinking behavior by comparing the PEth concentrations in whole blood with EtG in proximal hair segments of up to 5 cm length, which is used in routine analysis for DAA in Switzerland and in Germany.

Material and methods

Study design

Blood and corresponding hair samples were obtained from 136 persons undergoing DAA at the Department of Traffic Sciences of the Institute of Forensic Medicine at the University of Bern, Switzerland. Samples were collected between December 2014 and December 2015. Blood was taken on the same day as hair samples and was analyzed for PEth homologues 16:0/18:1 and 16:0/18:2 by a validated online-SPE-LC-MS/MS method [25]. Hair was analyzed for EtG by a modified LC-MS/MS method according to Kummer et al. [26]. Hair samples were stored at room temperature. Blood samples were stored at 4 °C prior to analysis and were analyzed one week after sampling at the latest.

Chemicals and materials

PEth 16:0/18:1 and PEth 16:0/18:2 were provided by Avanti Polar Lipids (Alabaster, USA). EtG and deuterated EtG were purchased from Lipomed (Arlesheim, Switzerland). Ammonium acetate, acetone and dichloromethane were obtained from Merck (Darmstadt, Germany).

2-Propanol was obtained from Fisher Scientific (Loughborough, UK), methanol (MeOH) was from Biosolve BV (Valkenswaard, Netherlands) and acetonitrile (MeCN) was supplied by Agros Organics (New Jersey, USA). Formic acid (98 %) was provided by Fluka (Switzerland). HPLC solvents were of gradient grade; all other solvents were of analytical grade.

H₂O dest. was produced in-house with a Milli-Q water system from Millipore (Billerica, USA).

Deuterated standards were synthesized in our laboratory from phosphatidylcholine PC 16:0/18:1 and phosphatidylcholine PC 16:0/18:2, respectively, and D₆-ethanol catalyzed by phospholipase D [1].

EDTA monovettes (volume 3.4 mL) were obtained from Sarstedt (Nümbrecht, Germany). Lithium-heparinized whole blood, which was used as blank blood, was donated by volunteers who were abstinent from alcohol for at least 4 weeks. Blank hair samples were donated from teetotalers.

Determination of PEth in whole blood

PEth was analyzed in 200 µL of whole blood by online-SPE-LC-MS/MS with a QTrap 3200 mass spectrometer (AB Sciex, Toronto, Canada). Analytical separation was performed by a Luna RP-C5 column, 50 mm x 2 mm, 5 µm particle size (Phenomenex, Brechbühler, Schlieren, Switzerland) by gradient elution using ammonium acetate (10mM)/MeCN (30:70, v/v) and 2-propanol. The precise details of the validated method can be found in [25].

Determination of EtG in hair

EtG was determined in hair with a limit of quantification (LOQ) of 3 pg/mg by a validated modified LC-MS/MS method according to [26] with a Ultimate 3000 LC-system (Dionex, Thermo Fisher Scientific, Reinach, Switzerland) coupled to a QTrap 5500 mass spectrometer (AB Sciex, Toronto, Canada). This method is used for routine analysis in the accredited laboratory (ISO 17025) of the Institute of Forensic Medicine Bern for samples from DAA according to SGRM guidelines [3].

Briefly, approx. 30 mg of hair was washed with 3 different solvents (4 mL each of 1. H₂O, 2. acetone, 3. dichloromethane) by subsequent shaking for 4 min and removal of the solvent between washing steps. After 1h of drying, the hair was grinded for 5 min (30 1/s) with a ball-mill (MM400, Retsch, Haan, Germany). The hair powder was extracted for 2 h by ultrasonication with 1 mL D5-EtG containing deionized H₂O. Further purification of the extract was achieved by SPE with Oasis Max cartridges (3cc/60 mg; Waters, Baden-Dättwil, Switzerland). The samples were analyzed in MRM mode with online preconcentration using a Hypercarb column, 3.0 x 2.1 mm, 5 µm (Thermo Fisher Scientific, Reinach, Switzerland), and after switching a two way valve, back-flow elution to the analytical column Zorbax Eclipse, 50 mm x 4.6 mm, 1.8 µm (Agilent, MSP Kofel, Zollikofen, Switzerland) and gradient elution. The following transitions were used: EtG: m/z 221/75 as quantifier and m/z 221/85 as qualifier. For the deuterated internal standard D5-EtG the transitions m/z 226/75 (quantifier) and m/z 226/85 (qualifier) were used.

Statistic evaluation

ROC analysis (receiver operating characteristic) was performed with GraphPad Prism 6 by classifying the PEth results into three groups depending on hEtG thresholds, suggested by the SOHT (hEtG ≥ 30 pg/mg: excessive alcohol consumption; 7 pg/mg ≤ hEtG < 30 pg/mg: moderate alcohol consumption; hEtG < 7 pg/mg: “non-contradicting to self-reported abstinence”) [10, 11].

Results

136 samples from persons undergoing DAA were analyzed. The controlled persons were between 19 and 78 years old (mean age: 42 ± 15 years), 88.2 % were male (120 male, 16 female).

The PEth and hEtG results were distributed into three groups: Excessive alcohol consumption, moderate alcohol consumption and sobriety (abstinence from alcohol) according to the thresholds suggested by the SOHT [10, 11] for hEtG. For PEth 16:0/18:1, the proposed thresholds (210 ng/mL and 20 ng/mL) were obtained from a Swedish study [27] and from results of drinking studies [14, 24, 25], meaning that PEth 16:0/18:1 values below 20 ng/mL (LOQ of the method) were rated as consistent with abstinence, and PEth 16:0/18:1 values above 210 ng/mL were classified to excessive alcohol consumption. For PEth 16:0/18:2 the following thresholds were used: PEth 16:0/18:2 ≥ 100 ng/mL: excessive alcohol consumption;

20 ng/mL \leq PEth 16:0/18:2 < 100 ng/mL: moderate alcohol consumption; PEth 16:0/18:2 < 20 ng/mL: consistent with abstinence.

With hEtG analysis, excessive alcohol consumption was detected in 37.5 % of the samples; and 44.1 % of the samples were consistent with abstinence. Moderate alcohol consumption was detected in 18.4 % of the samples. With PEth 16:0/18:1 elevated alcohol consumption was detected only in 22.8 % of the samples, but 58.1 % of the samples were already consistent with abstinence. Moderate alcohol consumption was detected in 19.1 % of the samples.

29 of 30 persons with PEth 16:0/18:1 \geq 210 ng/mL had hEtG \geq 30 pg/mg. Only one person was classified into the group of excessive alcohol consumers by PEth analysis whereas hEtG indicated moderate consumption (PEth: 450 ng/mL, hEtG: 29 pg/mg).

In 54 cases neither PEth nor hEtG was detected. In 8 cases PEth was $20 \leq$ PEth < 210 ng/mL, and hEtG was between 7 and 30 pg/mg, both markers showing moderate alcohol consumption.

Nine tested persons showed excessive alcohol consumption after analyzing hEtG, but PEth in blood was negative. There were no tested persons with negative hEtG values and PEth values above the threshold for excessive alcohol consumption of 210 ng/mL.

There was a little overlapping of different PEth and hEtG results between excessive and moderate alcohol consumption and between moderate alcohol consumption and abstinence. For example hEtG showed excessive alcohol consumption, but the corresponding PEth result showed moderate alcohol consumption (8.8 %), or hEtG showed moderate alcohol consumption, but PEth was not detectable (11.8 %). There were also tested persons with negative hEtG results, whereas PEth was detectable in blood, demonstrating moderate alcohol consumption (4.4 %). Thus, very recent alcohol consumption might have taken place, which could not be detected in hair, because of the gap of approximately 14 days that hair needs to grow out of the scalp before it can be cut. All results are summarized in detail in table 1.

As also the PEth homologue 16:0/18:2 was included in the column-switching LC-MS/MS method, this homologue was also compared to hEtG. Here a value of PEth 16:0/18:2 \geq 100 ng/mL was used as threshold to differentiate excessive from moderate alcohol consumption. PEth 16:0/18:2 values below 20 ng/mL (LOQ of the method) were regarded as consistent with abstinence. With these thresholds, the distribution of results obtained by PEth 16:0/18:1 were close to the results obtained by hEtG. With PEth 16:0/18:2 elevated alcohol consumption was detected in 22.8 % (hEtG: 37.5 %), abstinence was detected in 61.0 % (hEtG: 44.1 %) and moderate alcohol consumption was detected in 16.2 % (hEtG: 18.4 %) of the samples.

For PEth 16:0/18:1 and 16:0/18:2, the used thresholds to differentiate abstinence, moderate and excessive alcohol consumption were evaluated by ROC analysis. Using the software GraphPad Prism 6, the PEth results were classified into three groups depending on hEtG (figure 2). All tested persons with hEtG ≥ 30 pg/mg were assumed to have prolonged excessive drinking habits, persons with hEtG between 7 – 30 pg/mg were classified to moderate alcohol consumption and persons with hEtG < 7 pg/mg were assumed to be abstainers or at least there was no contradiction to abstinence.

Using the suggested “Swedish threshold” of PEth 16:0/18:1 ≥ 210 ng/mL for the differentiation of excessive from moderate alcohol consumption, there is a specificity of 98.8 % and a sensitivity of 58.8 %. With a specificity of 100 % at a threshold of 470 ng/mL, there is only a sensitivity of 45.1 %. For the differentiation of excessive from moderate alcohol consumption the PEth 16:0/18:1 threshold of 150 ng/mL resulted in the best sensitivity (70.6 %) and specificity (98.8 %) (figure 3 A).

For the differentiation of drinking habits (“non-contradicting self-reported abstinence” from “moderate alcohol consumption”) the PEth 16:0/18:1 threshold of 20 ng/mL (LOQ) resulted in the best compromise for sensitivity (67.1 %) and specificity (90.0 %). With a specificity of 100 % at a threshold of 87 ng/mL, there is only a sensitivity of 53.9 %. Using the LOD of our method of 10 ng/mL, there is a specificity of 86.7 % and a sensitivity of 71.1 % (figure 3 C). PEth 16:0/18:1 values between the suggested thresholds of 20 ng/mL and 150 ng/mL would pass for moderate alcohol consumption habits.

Using the suggested “threshold” of PEth 16:0/18:2 ≥ 100 ng/mL, there is a specificity of 98.8 % and a sensitivity of 58.8 %. With a specificity of 100 % at a threshold of 332 ng/mL, there would only be a sensitivity of 33.3 %. For the differentiation of excessive from moderate alcohol consumption the PEth 16:0/18:2 threshold of 96 ng/mL resulted in the best sensitivity (62.8 %) and specificity (98.8 %) (figure 3 B).

For the differentiation of abstinence from moderate alcohol consumption the PEth 16:0/18:2 threshold of 20 ng/mL (LOQ) resulted in the best compromise for sensitivity (63.2 %) and specificity (90.0 %). With a specificity of 100 % at a threshold of 93 ng/mL, there would only be a sensitivity of 44.7 %. Using the LOD of our method as threshold, which is 10 ng/mL, there is a specificity of 88.3 % and a sensitivity of 67.1 % (figure 3 D). PEth 16:0/18:2 values between the suggested thresholds of 20 ng/mL and 96 ng/mL would pass for moderate alcohol consumption habits.

Discussion

Although PEth and hEtG cover different time periods of alcohol consumption, PEth 16:0/18:1 (thresholds: 210 ng/mL and 20 ng/mL) and hEtG results are in agreement in 68 % of tested persons undergoing DAA. By comparing PEth 16:0/18:2 (thresholds: 100 ng/mL and 20 ng/mL) to hEtG, 66 % of the samples are in agreement.

Using the thresholds resulting from ROC analysis, results are in agreement in 72 % of the samples for PEth 16:0/18:1 (thresholds: 150 ng/mL and 20 ng/mL) (see table 1: numbers in line 2 and 3 in italics), for PEth 16:0/18:2 68 % of the samples are in agreement in comparison to hEtG (PEth 16:0/18:2 thresholds: 96 ng/mL and 20 ng/mL).

Of the tested persons, who were classified into the group with moderate alcohol consumption habits ($7 \text{ pg/mg} \leq \text{hEtG} < 30 \text{ pg/mg}$), only one person had a PEth 16:0/18:1 value above the threshold of 150 ng/mL (sensitivity: 70.6 %, specificity: 98.8 %) resulting from ROC analysis. However, the hEtG result is very close to be classified as excessive alcohol consumption (hEtG: 29 pg/mg and PEth: 450 ng/mL). These results show that all PEth values above this threshold are representing excessive alcohol consumption habits with a specificity of 98.8 %, and that the PEth 16:0/18:1 threshold for differentiation of excessive and moderate alcohol consumption can be lowered to 150 ng/mL for persons undergoing DAA.

There is a relatively high number of persons undergoing DAA, who showed excessive alcohol consumption habits (37.5 % detected by hEtG analysis, 22.8 % detected by PEth analysis). The reason for these findings could be that alcohol consumption habits were assessed soon after withdrawal of the driver's license, and drinking habits might not have been changed in the meantime.

The reasons for minor discrepancies between PEth and hEtG results are due to the different detection windows of the matrices and analytes utilized (blood: PEth, hair: EtG). As PEth has a shorter detection window (ca. 2 – 4 weeks) than hEtG (several months depending on the hair length) [8], changes in drinking behavior can be detected earlier by PEth than by hEtG analysis alone. Furthermore, PEth can be directly detected in blood a few hours after alcohol ingestion, whereas EtG first needs to be incorporated into the hair bulb. As the scalp hair growth rate can vary from 0.6 – 1.5 cm/month [28] with an average rate of approximately 1 cm/month, and an additional time period for hair growth (of 10 – 14 days) has to take place until the incorporated EtG is found in the proximal segment of head hair, a segment of 5 - 6 cm length corresponds to a retrospective time window of approximately 6 months [3].

Therefore, PEth shows changes in drinking habits earlier than hEtG. Withdrawal or relapse to drinking can be shown directly after drinking or cessation of drinking by PEth analysis. Additional PEth analysis during DAA increases the sensitivity for uncovering alcohol consumption, although a single blood sampling cannot cover a six months period – which is the approximated period covered by EtG analysis with a 5 cm proximal hair segment.

Conclusion

PEth is a valuable additional marker for DAA and helps to improve diagnostic information. As PEth has a shorter detection window (ca. 2 – 4 weeks) than hEtG (of a 5 cm proximal hair segment: approximately 6 months), changes in drinking behavior can be detected earlier by PEth in blood than by hEtG analysis alone. Another advantage of PEth analysis is that unlike hair, blood is always available in sufficient amounts.

Therefore, we strongly recommend including the two most abundant PEth homologues in human blood, PEth 16:0/18:1 and PEth 16:0/18:2, in routine analysis for DAA. PEth should be analyzed in 2 – 3 blood samples over 6 months, as elevated PEth concentrations would demonstrate a lack of abstinence which might cause, prior to re-granting the driver's license, a longer withdrawal or, after re-granting the driver's license, a new withdrawal for a non-compliant person.

Tables and figures (in order of appearance in the text)

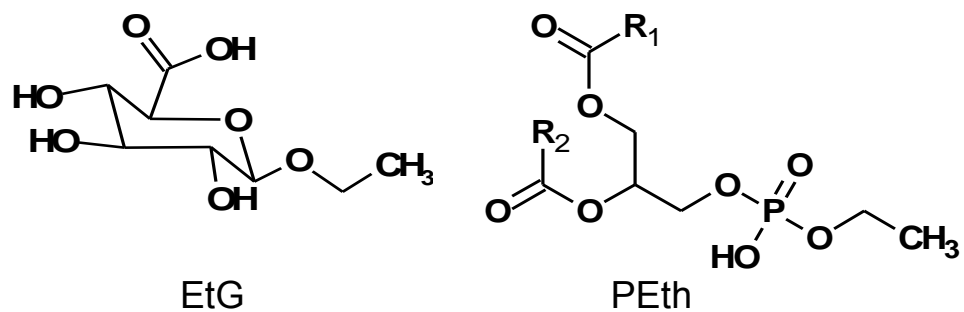


Figure 1. Structural formulas of ethyl glucuronide (EtG) and phosphatidylethanol (PEth);
R₁ and R₂: fatty-acyl-rests.

Table 1. Classification of alcohol consumption habits using hEtG and PEth 16:0/18:1 from 136 tested persons undergoing DAA.

For PEth the following thresholds were used: PEth < 20 ng/mL: abstinence*; PEth ≥ 210 ng/mL, and after ROC analysis PEth ≥ 150 ng/mL: excessive alcohol consumption.

Drinking habits		PEth ≥ 210 ng/mL; PEth < 20 ng/mL	PEth ≥ 150 ng/mL; PEth < 20 ng/mL
hEtG	PEth	in % (number of samples)	in % (number of samples)
Excessive	Excessive	22.1 (30)	26.5 (36)
Excessive	Moderate	8.8 (12)	4.4 (6)
Moderate	Excessive	0.7 (1)	0.7 (1)
Moderate	Moderate	5.9 (8)	5.9 (8)
Abstinence*	Moderate	4.4 (6)	4.4 (6)
Moderate	Abstinence*	11.8 (16)	11.8 (16)
Abstinence*	Abstinence*	39.7 (54)	39.7 (54)
Excessive	Abstinence*	6.6 (9)	6.6 (9)
Abstinence*	Excessive	0 (0)	0 (0)

*Non-contradicting self-reported abstinence according to SOHT [11]

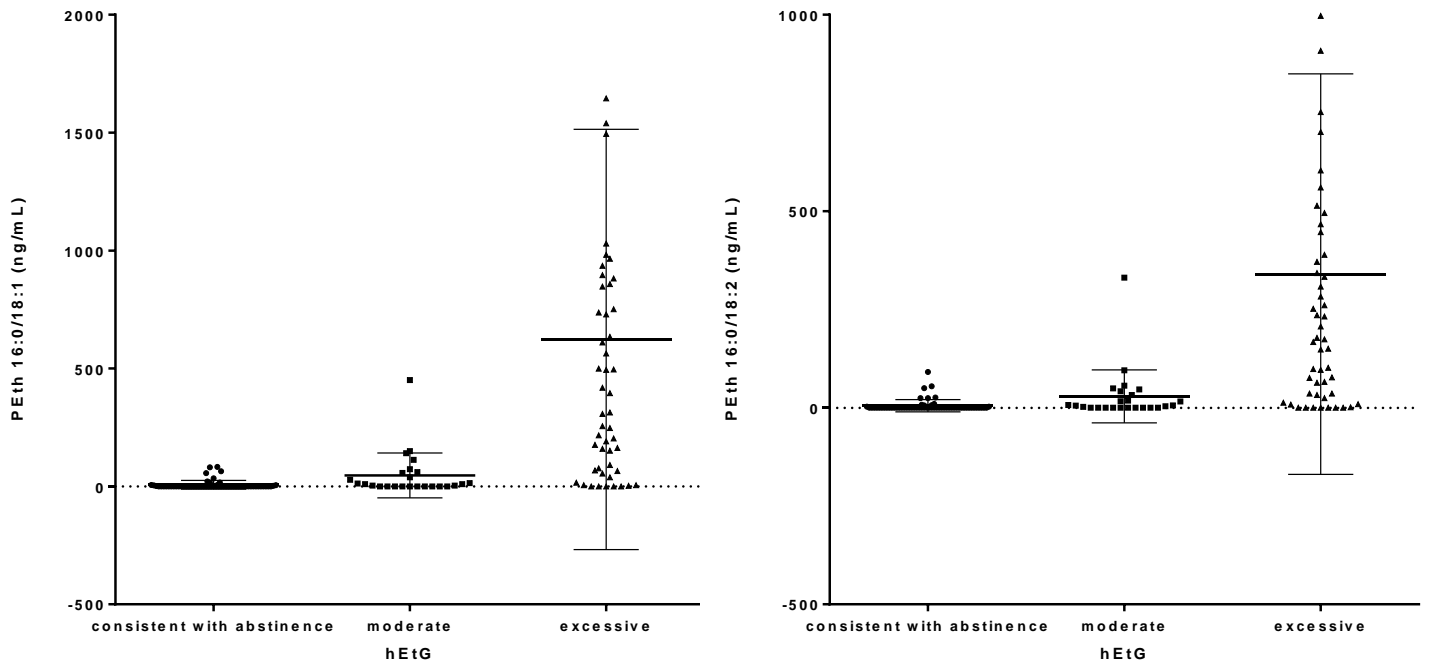


Figure 2. Classification of PEth 16:0/18:1 and 16:0/18:2 results in comparison to hEtG (Consistent with abstinence: hEtG < 7 pg/mg; moderate: $7 \leq$ hEtG < 30 pg/mg; excessive: hEtG \geq 30 pg/mg). Three data points (PEth 16:0/18:1 and PEth 16:0/18:2, respectively) are outside the axis limit in the column of excessive hEtG (1. PEth 16:0/18:1: 2155 ng/mL, PEth 16:0/18:2: 1495 ng/mL; 2. PEth 16:0/18:1: 3545 ng/mL, PEth 16:0/18:2: 1945 ng/mL; 3. PEth 16:0/18:1: 4870 ng/mL, PEth 16:0/18:2: 2685 ng/mL).

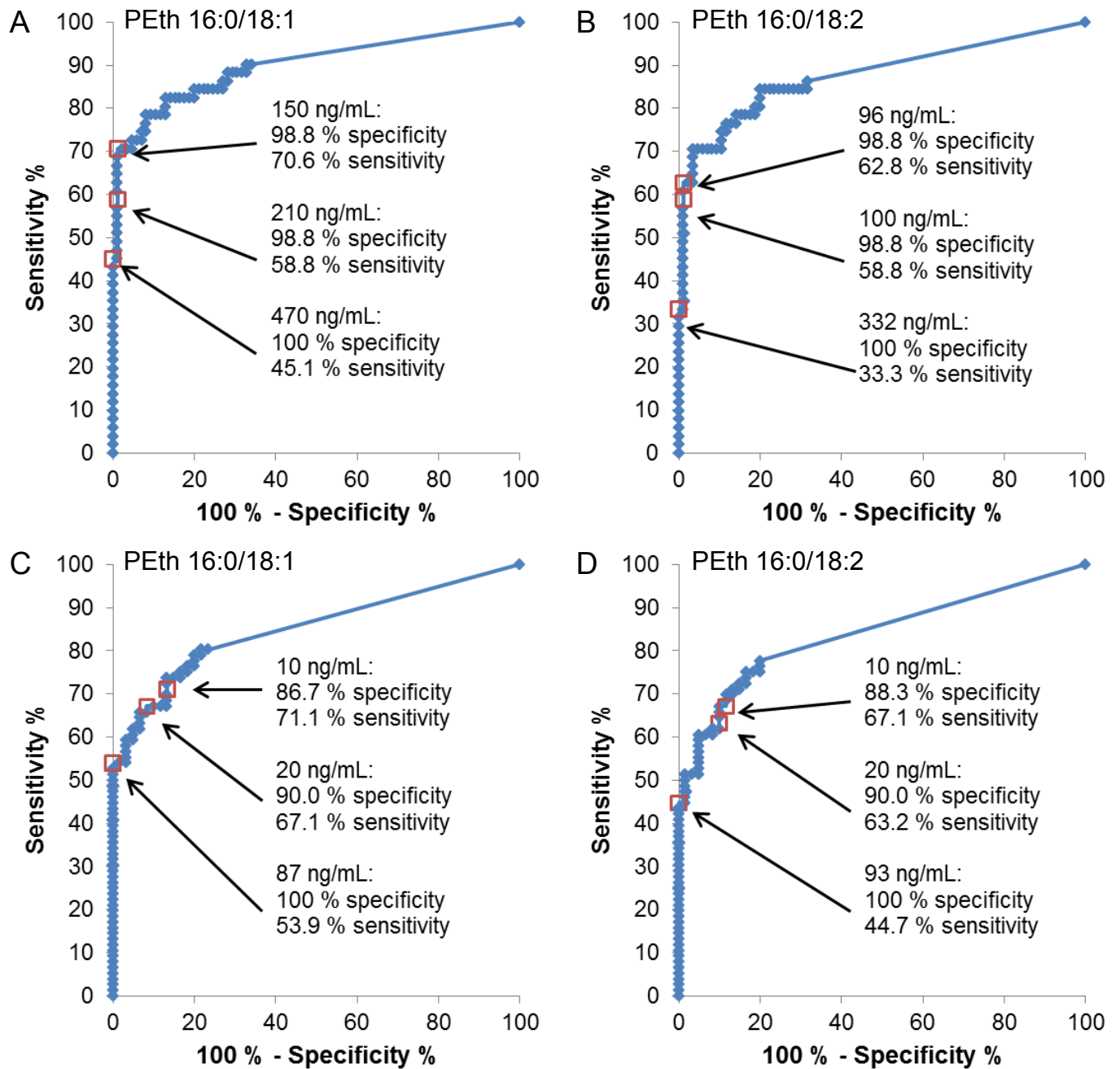


Figure 3. ROC analysis for PEth vs. hEtG. **A:** PEth 16:0/18:1 vs. hEtG to differentiate excessive from moderate alcohol consumption (AUROC 0.899, P value < 0.0001). **B:** PEth 16:0/18:2 vs. hEtG to differentiate excessive from moderate alcohol consumption (AUROC 0.876, P value < 0.0001). **C:** PEth 16:0/18:1 vs. hEtG to differentiate abstinence from moderate alcohol consumption (AUROC 0.85, P value < 0.0001). **D:** PEth 16:0/18:2 vs. hEtG to differentiate abstinence from moderate alcohol consumption (AUROC 0.84, P value < 0.0001).

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