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False Positive Results of Phosphatidylethanol (PEth) Quantitation in Dried Blood Spots (DBS): The Influence of Alcohol Vapors

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Abstract: The role of phosphatidylethanol (PEth) as an alcohol consumption marker is increasing in clinical and forensic medicine. During the COVID-19 pandemic, the role of hygiene increased, and it became common practice to use disinfectants almost everywhere. This paper highlights a possible source of false positive results (by the vapors of alcohols during the blood spot drying process) in dried blood spots (DBS) by LC-MS/MS quantitation of PEth. To achieve this, the PEth quantitation method was validated according to FDA guidelines. Additionally, the synthesis of phosphatidyl derivatives by phospholipase D (PLD) in the presence of methanol and 2-propanol vapors during the DBS process was determined. Each PEth-negative sample from a healthy male patient incubated in the presence of ethanol vapor becomes PEth-positive. After 4 h of DBS drying, teetotalers become "moderate drinkers". It is necessary to avoid using alcohol-containing disinfectants in treatment rooms, where DBS is sampled.

Keywords: phosphatidylethanol; alcohol biomarker; forensics; clinical medicine; LC-MS/MS

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1. Introduction

A direct marker of alcohol consumption, phosphatidylethanol (PEth), is a group of abnormal phospholipids formed by phospholipase D (PLD) in the presence of ethanol [1]. PEth consists of the same glycerophospholipid central chain, and two side chains of various long-chain carboxylic acids. They are classified according to the fatty acid residues in their composition. Nowadays, in alcohol drinkers' blood, approximately 50 fatty acid combinations were identified in PEth composition [2]. The most common combination of fatty acids includes palmitic acid (16 carbon atoms and 0 double-bonds/16:0) and oleic acid (18 carbon atoms and 1 double bond/18:1), approximately 40% of the total amount [3,4]. This homolog is generally used for quantification in both forensic and clinical applications [5–9].

PEth concentrations provide information about a subject's drinking habits, which could be used in different spheres of life, e.g., alcohol-withdrawal treatment settings [10], driving aptitude assessments [11], or transplantology [12,13]. Generally, drinkers could be classified into three groups: teetotalers, moderate drinkers, and excessive consumers. This separation requires two thresholds: the lower and the upper. It is a common practice to use levels of 20 and 200 ng/mL, respectively. However, the thresholds may differ depending on the country or region [4,5,14].

Nowadays, using LC-MS/MS for the quantification of PEth homologs is a common practice due to high sensitivity and selectivity [2,5–9]. The easiest way of sampling blood

to quantify PEth is by using dried blood spot (DBS) technology. It requires lesser volumes in comparison with venous and does not require special medical staff for sampling [15]. Compared with whole blood, in which PEth is only stable at −80 °C, in DBS PEth is stable for a month at room temperature [16,17]. The drying process removes water and inactivates enzymes, resulting in improved stability of the PEth. However, it is still unstable, until the drying process is completed [17].

Alcohol-containing (including ethanol) antiseptics are used to disinfect workplaces in treatment rooms or to sanitize the hands of medical staff [18,19]. Upon finishing sample collection, medical staff usually disinfect their working place with one kind of antiseptic (including alcohol-containing). This study was undertaken to investigate the possibility of PEth formation (PEth 16:0/18:1) via a reaction with alcohol vapors during the DBS drying process and find out if any other products of phosphatidylcholine hydrolysis by PLD and volatile alcohol vapors may be formed.

2. Materials and Methods

2.1. Reagents

PEth reference material containing palmitic acid and oleic acid (palmitoyl-oleoyl-sn-glycero-3-phosphoethanol, PEth 16:0/18:1) was obtained as a chloroform solution from Avanti Polar Lipids (Alabaster, AL, USA). The internal standard (IS) deuterated PEth 16:0/18:1 (palmitoyl-oleoyl-sn-glycero-3-phosphoethanol-D5, PEth 16:0/18:1-D5) was purchased from Chiron AS (Chiron, Trondheim, Norway). LC or analytical grade acetonitrile, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-2-propanol, and ammonium acetate were obtained from Merck (Darmstadt, Germany). Filter paper Whatman 903 was purchased from Cytiva (Cytiva, Bangalore, India). Ultrapure water was supplied by an Adrona Crystall purification system (Adrona, Riga, Latvia).

2.2. Blood Sampling

The authentic blood samples were collected at the City Clinical Hospital N4 (Moscow, Russia) using EDTA tubes from six healthy patients with a normal constitution, who had not consumed alcohol in the last six months (tested with AUDIT-C [20]). Local Ethics Committee of I.M. Sechenov First Moscow State Medical University (Sechenov University) approved this study (protocol #01-20 of Local Ethic Committee meeting on 22 January 2020)

2.3. Sample Preparation

Blood lipids were extracted according to the procedure proposed by Aboutara et al., with some minor modifications [8].

Working solutions containing PEth 16:0/18:1 were prepared to spike the calibrators (10, 50, 100, 500, 1000, 2000 ng/mL) and the quality controls (QCs) (10, 30, 200, and 1500 ng/mL). After the standard solution was spiked, each sample was gently vortexed for 30 m. Blood spots (50 µL) were pipetted onto Whatman 903 filter paper using an Eppendorf pipette. The blood spots were left to dry for at least 3 h at room temperature and then stored in a Ziploc bag with a desiccant bag in the dark at room temperature for further sample analysis. DBSs were cut out as a whole and put into a disposable glass tube. MeOH (1 mL) containing PEth 16:0/18:1-D5 (100 ng/mL) was added. After the disposable glass tubes were sealed, they were shaken for 45 min on an orbital shaker. Then, 800 µL of MeOH was transferred into a V-vial and evaporated at 30 °C using an Eppendorf concentrator. The residue was reconstituted in 200 µL of acetonitrile:isopropanol 1:1 and vortexed for 30 s.

To simulate the formation of phosphatidyl in DBS during drying, fresh Peth-negative blood (50 µL) was pipetted onto Whatman 903, cut out as a whole, and was hanged in a sealed jar (with nominal volume of 1 L), where 1 µL of alcohol was previously pipetted. A 1 µ/L concentration of alcohol vapors, the theoretical concentration of whole alcohol which was used for the sanitary treatment [19] of a typical treatment room in Russia, would be vaporized at once. Jar was incubated, shielded from direct sunlight, and kept at room

temperature for 4 and 8 h. After incubation, lipids from discs were prepared the same way as described above, but with the use of MeOH (1 mL) without IS.

2.4. Instrumentation for Qualification Phosphatidyl Derivatives

For the qualification of phosphatidyl derivatives, a Waters ACQUITY UPLC (Waters, Milford, MA, USA) system coupled with Thermo QExactive mass spectrometer (Thermo, Bremen, Germany) was used. Separation was performed on an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm) in gradient mode at a 0.4 mL/min flow rate. The mass spectrometer was operated in the PRM mode with 30 V CE and negative ESI (4.5 kV). Data were processed using XCalibur software. Identification was conducted similarly to the procedure proposed by Gnann [2] by high-resolution MS1 for phosphatidyl derivatives (with fatty acid configuration 16:0/18:1 and 16:0/18:2) and MS2 fragmentation pattern, see Table S1 and Figure 1.

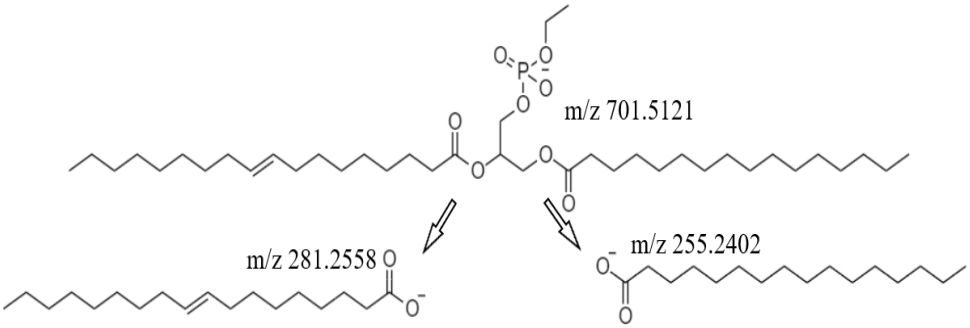

Figure 1. Example of fragmentation pattern for PEth 16:0/18:1.

2.5. Instrumentation and Validation Procedure for Quantification PEth in DBS

An Agilent UHPLC-MS-MS instrument consisted of an Agilent 1290 Infinity HPLC system interfaced to a triple quadrupole Agilent 6460 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Separation was achieved with a Phenomenex Kinetex® C18 LC Column (2.6 µm, 50 × 3 mm). The temperatures of the column and the sample were maintained at 50 °C and 4 °C, respectively. The mobile phase, at a flow rate of 0.8 mL/min, included water:acetonitrile 1:1 (A) and isopropanol:water 10:1 (B) both containing 5 mmol/L ammonium formate, was applied in the following gradient elution: 0–3 min, 55% B to 70% B; 3–5 min, held as 70% B; after 5.10 min held as 55% B. The total run time for the analysis was 9 min (with re-equilibration). The injection volume was 4 µL. The mass spectrometer was operated in negative MRM mode with ESI parameters set at: gas temperature 300 °C, gas flow 7 L/min, nebulizer 40 psi, sheath gas heater 400 °C, sheath gas flow 12 L/min, capillary voltage 3.5 kV, VCharging 500 V. Parameters of the ion source were optimized manually in order to maximize signal-to-noise ratio (SNR) of the analytes. MRM transitions for qualitative analysis of the analyte and the IS were 701.5/255.4 (CE 35) and 706.5/255.4 (CE 35), respectively. Qualitative transitions for the analyte and the IS were 701.5/281.4 (CE 33) and 706.5/281.4 (CE 33), respectively. All dwell times were set to 100 ms. MRM transitions were optimized using Mass Hunter Optimizer software. Data were processed with Mass Hunter QQQ Quantitative Analysis software.

The developed UHPLC method was validated regarding selectivity, recovery, matrix effects, carry-over, the limit of quantification (LOQ), linearity, intra-day, and inter-day precision for three consecutive days, stability, and accuracy according to the FDA Guidance for Industry with minor modifications [21]. The samples were extracted under optimized conditions similar to the extraction protocol mentioned in the sample preparation subsection above, then analyzed using the developed method. All samples and standards were analyzed in two independent runs, except samples for determination accuracy and precision—they were analyzed in six independent runs. The LLOQ was set to 10 ng/mL due to there being no need to measure the quantity of PEth 16:0/18:1 below the first threshold (20 ng/mL). Long-term stability was not evaluated because the samples were

analyzed immediately within the current working day. Several scientific groups reported that Peth-positive DBS are highly stable at room temperature (up to 9 months) [16,17]. Hematocrit inflicts measure results on PEth because PEth is generally located in erythrocyte cell membranes [22]. However, the used decision limits (thresholds mentioned above) for PEth do not account for differences in the hematocrit, which is why we did not measure and normalized results on hematocrit.

3. Results

3.1. Synthesis of Phosphatidyl Derivatives in Alcohol Vapors

To find out if traces of alcohol (including ethanol) vapors can affect the quantification of chronic ethanol consumption marker PEth 16:0/18:1, we dried DBS in the presence of vapor as described above. Methanol, ethanol, 1-propanol, 2-propanol, 1-butahol, 2-butanol, and 2-methyl-2-propanol are alcohols, which were used for the synthesis experiment. Via PLD from blood during the DBS drying process were synthesized phosphatidyl derivatives with methanol (PMeth 16:0/18:1 and PMeth 16:0/18:2), ethanol (PEth 16:0/18:1 and PEth 16:0/18:2), and 2-propanol (PiPro 16:0/18:1 and PiPro 16:0/18:2).

3.2. Quantification of PEth

Optimized chromatographic conditions were achieved after several trials with acetonitrile, methanol, isopropanol, and water in different proportions for the mobile phase. The mobile phase containing water:acetonitrile 1:1 and 2-propanol:water 10:1, both containing 5 mmol/L ammonium formate, the constant flow rate of 0.8 mL/min and gradient elution at the fixed column temperature of 50 °C were deemed the optimal separation and best performance conditions for the determination of PEth 16:0/18:1 in spiked blood samples.

The developed method was validated regarding selectivity, recovery, matrix effects, carry-over, the limit of quantification (LOQ), linearity, intra-day and inter-day precision, stability, and accuracy (Tables S2 and S3). These validation parameters enabled us to investigate the suitability of the method for our study's goal and routine analysis.

There was no interference from other components in blood at the retention times of the analytes. See Figure 2, PEth 16:0/18:1 RT 2.4 min.

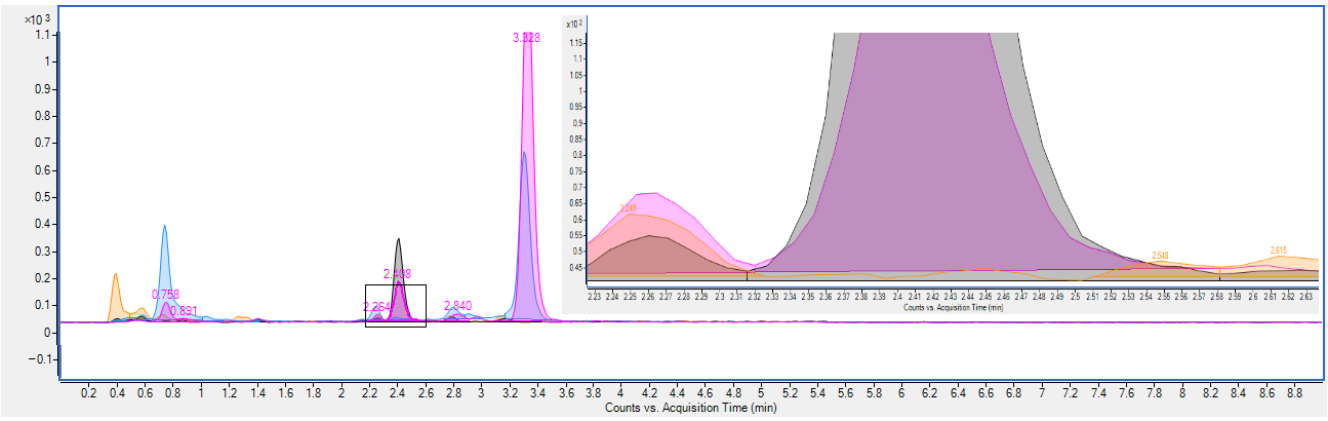

Figure 2. Blank (blue 701.5/255.4 and gold 706.5/255.4) and LLOQ (magenta 701.5/255.4 and grey 706.5/255.4) sample chromatograms.

The extraction recovery rates were higher than 74%. The matrix effect was fully compensated by IS. The six-point calibration curve exhibited a linear correlation between concentration and peak area. No carry-over influence on the quantification at the LLOQ level was identified. Calibration data indicated the linearity ($R^2 > 0.99$) of the detector response for PEth 16:0/18:1 from 10 to 2000 ng/mL, see Figure 3.

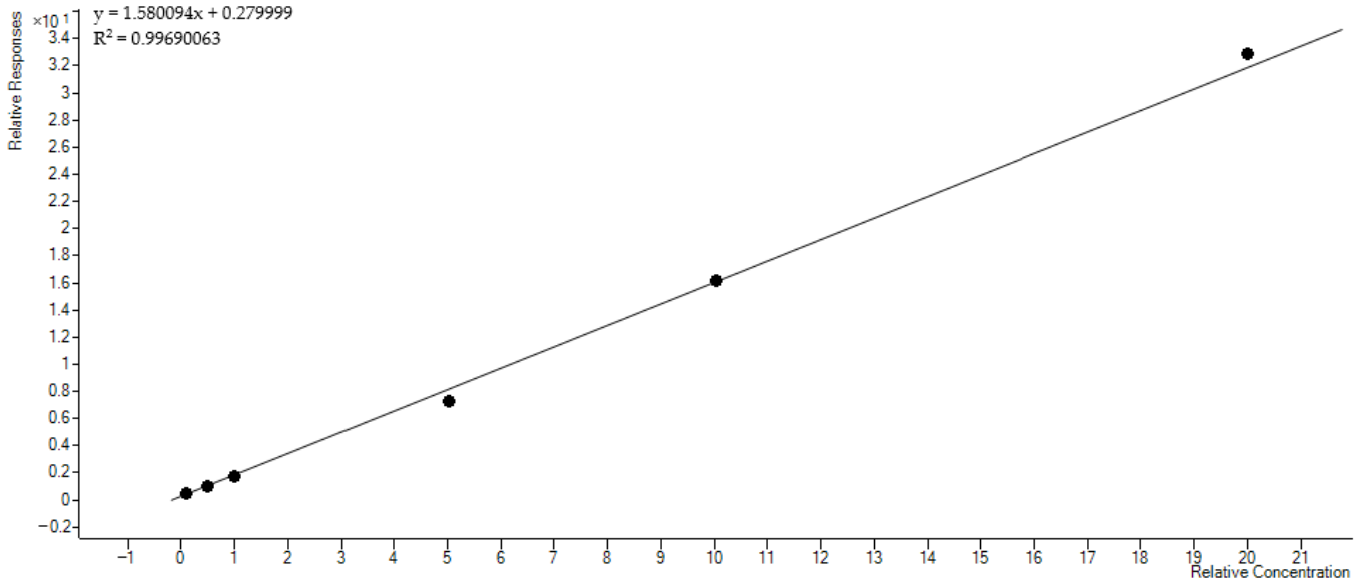

Figure 3. Calibration curve.

The SNR at the LLOQ level of concentration was higher than 50. The intra- and inter-day variation for this study were determined with relative standard derivation (RSD) and were both lower than 9.89%. The analysis was conducted three times on three different days, and each run was repeated six times. The intra-day RSD for the replicates was between 3.01 and 8.90%, and the RSD for the day-to-day replicates was between 4.02 and 9.89%. The method accuracy was between 92.1 and 118.8% (for LLOQ). Samples were stable from the moment of sample preparation till UHPLC-MS/MS analysis.

Quantitative PEth 16:0/18:1 results of the six replicates each for 4 and 8 h incubation are presented in Table S4. Blood used for the experiment was from six healthy males with normal constitutions. The mean age of the patients was 26.7 (range 24–29). None of the blood samples used for this experiment were positive for ethanol or PEth. Samples incubated with vapors of ethanol for 4 and 8 h were positive with an average concentration of 88.59 ± 25.49 ng/mL and 180.88 ± 91.40 ng/mL, respectively.

4. Discussion

In this research, we studied phosphatidyl derivatives synthesis by PLD in the presence of alcohol vapors during the DBS drying process. The presence of μg ethanol as vapor in a room where DBS is drying leads to false positive results for PEth testing (teetotalers become moderate drinkers) in 4 h. For DBS disk drying in such conditions for 8 h, 66% of teetotalers became excessive consumers with a PEth 16:0/18:1 concentration > 200 ng/mL. During sampling and further drying of DBS, we need to abstain from using fluids and materials containing ethanol. After drying, PLD inactivates and further incubation does not depend on the amount of ethanol in the blood sample [17,23–25]. Beck et al. suggested a more applicable method of sampling using PLD inhibitors—for instance, $NaVO_3$— however, it complicates the process as PLD inhibitors must be included inside the blood collection tubes or on an internal surface of the volumetric capillary. Despite this, the use of inhibitors allows the interruption of the synthesis of the marker with PLD and alcohol contained in the blood samples [26]. Unfortunately, nowadays it is impossible to use PLD inhibitors routinely due to the lack of sampling devices coated with a PLD inhibitor, thus we need to notice all sources of false positive results and exclude them in time. The possibility of synthesizing other phosphatidyl derivatives with methanol and isopropanol has been shown, thus they should also be removed since each chemical modification of the sample leads to errors that can potentially affect the result of the study. Since PEth is considered a marker for use in the field of forensic or clinical medicine [5–9], even the probability of an error should be taken seriously.

5. Conclusions

The developed and validated UHPLC-MS/MS method for PEth 16:0/18:1 quantitation in DBS in general, complies with the FDA Guidance for Industry [21]. Experiments with drying blood spots in alcohol vapor showed the influence on the PEth quantitation which cannot be ignored (the source of false positive results). In addition, the synthesis of methanol and 2-propanol phosphatidyl derivatives was presented via drying blood spots in the presence of vapor of corresponding alcohol. Alcohol-containing means for sanitizing workplaces where DBS is drying should be removed due to influence on the sample and as a consequence on the PEth quantitation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9090250/s1, Table S1: precursor ion m/z of phosphatidyl homologs and products ion m/z corresponding to fatty acids; Table S2: method validation I; Table S3: method validation II; Table S4: quantitation PEth 16:0/18:1 in DBS, which was drying in ethanol vapors.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of I.M. Sechenov First Moscow State Medical University (Sechenov University) protocol #01-20 of Local Ethic Committee meeting on 22 January 2020.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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