The detection of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol in human dried blood spots

Joseph Jones,* Mary Jones, Charles Plate and Douglas Lewis

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Introduction

Alcohol abuse is an illness that has monumental consequences on people’s every day lives, their physical and mental health and the economies of nations. The National Epidemiologic Survey on Alcohol and Related Conditions reported that 8.5% percent of surveyed adults in the United States satisfied the definition for an alcohol use disorder.1 Healthy People 2010, a national health objective, outlined a goal to “reduce substance abuse to protect the health, safety, and quality of life for all, especially children”.2 An objective, sensitive, specific and long-term direct biomarker is needed to monitor ethanol abstinence for a variety of demographics, including, but not limited to, recovery program participants, organ transplant recipients and pregnant mothers.

Current devices to screen for excessive and prolonged use of alcohol consist of questionnaires that rely on subjective self-reports, which may be used with or without alcohol biomarkers. Indirect alcohol biomarkers measure phenomena that result from repeated ethanol exposure. Traditional indirect biomarkers such as mean corpuscular volume (MCV) and γ-glutamyl transferase (GGT) have insufficient sensitivity and specificity to be used as screening tests in unselected patients.3,4 Newer indirect alcohol biomarkers such as carbohydrate-deficient transferrin (CDT) and the early detection of alcohol consumption (EDAC) test are the most widely used biomarkers in the United States.3 Indirect markers of alcohol are not optimal for use in the general population because age, gender, organ pathologies and pregnancy affect the final outcome.3,4

Direct alcohol biomarkers measure ethanol itself or a metabolite produced only in the presence of ethanol. Currently, the measurement of ethanol in blood, breath and urine is the most widely used test for alcohol. With a very short detection window (1 hour per drink) it is ideal for traffic safety and under the influence monitoring. Ethyl glucuronide (ETG) and ethyl sulfate (ETS) detection using urine offers a middle ground of detection with a window of up to 80 hours.5 ETG is subject to post-collection synthesis and degradation, which diminishes its overall utility in a general population.6,7 The assay is very sensitive to unintentional exposure to ethanol, such as mouthwash and waterless sanitizer, resulting in detectable levels in urine.8,9 Misuse of the assay initiated multiple civil actions10–12 as well as a SAMHSA advisory concerning the use of urine ETG results to initiate disciplinary action.13 ETG detection in hair is showing promise as a long-term alcohol biomarker, but as of yet is not in widespread use.14,15 Fatty acid ethyl esters (FAEEs) detection in meconium is currently the standard for identifying newborns exposed to alcohol in utero but specimen availability, low sensitivity and post-collection synthesis limit its utility.16 The detection of FAEE has been reported in hair but post-collection synthesis limits the usefulness of this assay as well.17

Phosphatidylethanol (PEth) is a group of abnormal phospholipids that are formed in the presence of ethanol and phospholipase D.18–21 Once formed and incorporated into the erythrocytes, the half-life has been determined to be approximately 4 days which provides an extended window of detection of alcohol exposure.22 Two fatty acid moieties contained in the structure determine the specific isomer of PEth with the palmityl/oleoyl species being the most prevalent in the blood of heavy drinkers.23,24 Previous reports in the literature utilize high-pressure liquid chromatography (HPLC) with an evaporative light-scattering detector (ELSD).25–28 The ELSD detects all PEth

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isomers but has limited sensitivity when compared to a tandem mass spectrometry (MSMS) method that is focused on a single isomer.\textsuperscript{24} A comparison of ELSD and MSMS PEth whole blood methods was reported with excellent correlation at lower concentrations but diverged as the concentrations exceeded 2000 ng mL\textsuperscript{-1}.\textsuperscript{24} The reported limit of detection for the MSMS method was 20 times lower than the ELSD method. It would appear that while the ELSD method proved to have better correlation with various levels of excessive drinking, the MSMS method appears to be a better tool for early relapse detection and abstinence verification.

All of the previously mentioned PEth methods require a venipuncture blood collection. Venipuncture is expensive, invasive and impractical in a nonclinical setting. The purpose of this paper is to demonstrate a fully validated high pressure liquid chromatography-tandem mass spectrometry (LCMSMS) method for the detection of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol (POPE) using dried blood spots. The collection of dried blood spots has the advantage of being less-invasive and not requiring the services of a skilled collector, making collection feasible in a nonclinical setting, while maintaining sufficient sensitivity for verification of ethanol abstinence and early detection of relapse.

Experimental

Chemicals, reagents and materials

The internal standard, 1,2-dioleoyl-sn-glycero-3-phosphopropanol (PPro), was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The PEth of interest, POPE, was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). All reagents (ACS grade) and all solvents (HPLC grade) were purchased from Thermo-Fisher (Hanover Park, IL, USA). Dried blood spots were prepared on Whatman 903 Specimen Collection Paper (Florham Park, NJ, USA). A McGill Basic Series GEM 1/8” (3.175 mm) Round Hand Held Punch was purchased from Whatman, Inc (Sanford, ME, USA).

Preparation of calibration standards and quality control samples

Stock solutions of PPro and POPE were prepared in chloroform at a concentration of 100 µg mL\textsuperscript{-1}. Using different lots of POPE stock solutions, working solutions (1 µg mL\textsuperscript{-1}) were prepared by further dilution with mobile phase A (20% 2 mM ammonium acetate : 58% acetonitrile : 22% isopropanol). An internal standard working solution (PPro, 5 ng mL\textsuperscript{-1}) was prepared by appropriate dilution with mobile phase A. Using different lots of working solutions, a calibrator (20 ng mL\textsuperscript{-1}) and a set of controls (0, 8, 25, and 100 ng mL\textsuperscript{-1}) were prepared by spiking negative blood with an appropriate amount of POPE working solution. Dried blood spots were prepared by applying 30 µL of each calibrator and control to the Whatman 903 Specimen Collection Paper. The paper was allowed to dry for at least 3 hours according to the manufacturer’s instructions.

Specimen preparation

Using the McGill Round Hand Held Punch, three punches from the calibrator, controls and specimens were placed in 13 × 100 disposable glass culture tubes. To each tube, 50 µL of PPro internal standard was added (12.5 ng mL\textsuperscript{-1}). Methanol (0.5 mL) was added to each tube, covered, and shaken at room temperature for 1 hour. The methanol was transferred into a clean 13 × 100 disposable glass culture tube and evaporated under a stream of nitrogen at 60 °C. The residue was reconstituted in 100 µL of mobile phase A and analyzed by LCMSMS.

LCMSMS conditions

The specimens were analyzed using an Agilent Technologies 1200 system that consisted of a G1367D autosampler, a G1379B degasser, a G1312B binary pump, and a G1310 isocratic pump (Wilmington, DE, USA). Separation was achieved using an Agilent Zorbax Eclipse Plus (50 mm × 2.1 mm, 1.8 µm particle size) C-8 column held at 30 °C in a G1316B Thermostatted Column Compartment (Wilmington, DE, USA). Using a flow rate of 0.250 mL min\textsuperscript{-1}, the solvent system was a gradient that consisted of A (20% 2 mM ammonium acetate : 58% acetonitrile : 22% isopropanol) and B (60% acetonitrile : 40% isopropanol). The solvent program held B at 0% from 0.0 min to 5.0 min. Solvent B was increased to 100% between 5.0 min and 5.1 min and held at 100% until 7.0 min. Solvent B was decreased to 0% at 7.0 min and held at 0% until 11.0 min. The detector was an Agilent Technologies 6460 tandem mass spectrometer using electro-spray ionization (ESI) in the negative mode. The capillary voltage was set at 3500 V, the nozzle voltage set at 500 V and the desolvation gas (nitrogen) was heated to 300 °C with a flow rate of 5 L min\textsuperscript{-1}. The sheath gas (nitrogen) was heated to 250 °C and delivered at 11 L min\textsuperscript{-1} (Wilmington, DE, USA). The internal standard (PPro) was monitored using the m/z 741.5 > 281.2 transition. The m/z 701.5 > 281.2 (quantitation ion) and m/z 701.5 > 255.1 (qualifying ion) transitions were used to monitor POPE. All three transitions used a fragmentor voltage of 130 V and a collision energy of 30 V. All data were processed using a MassHunter B.02.01 (Wilmington, DE, USA).

Identification criteria

The identification criteria used for this procedure included four components: retention time, signal to noise, baseline resolution and relative ion intensity. The retention time of each analyte was required to be within 0.2 min of the calibrator. A signal to noise of greater than 3 : 1 was required of each ion chromatogram. A minimum of 90% return to baseline was required to consider a peak to be adequately resolved from a co-eluting peak. The relative ion intensity of the product ions for each analyte (mass ratio) was required to be within 20% of the corresponding relative ion intensity of the calibrator.

Method validation

The method was validated according to the recommendations of commonly accepted guidelines.\textsuperscript{29–33} The following parameters were evaluated: limit of detection (LOD), limit of quantitation (LOQ), linear range, carryover potential, selectivity, accuracy, precision, extraction efficiency, matrix effect, stability of extracts on the autosampler, and stability of specimens during freeze-thaw conditions. Additionally, the potential of post-collection synthesis was assessed for specimens containing significant blood
The extraction efficiency and matrix effect were determined using procedures defined by Matuszewski. Three sets of controls were prepared over four concentrations with five replicates each. The replicates were prepared with 5 different lots of negative blood. The concentrations analyzed were prepared at 20, 50, 100 and 200 ng mL\(^{-1}\). The first set was unextracted controls reconstituted in mobile phase A. The second set was negative blood spot extracts fortified with POPE that were subjected to the extraction procedure. The third set was negative blood spot controls fortified with POPE that were subjected to the extraction procedure. The extraction efficiency for each analyte is expressed as the ratio of the average peak area in set 3 to set 2. The matrix effect for each analyte is defined as the ratio of the mean peak area of set 2 to set 1. The relative matrix effect is defined as the precision (\%CV) of the standard line slopes constructed from set 3.

The stability of prepared extracts was assessed by the re-analysis of a control set (20, 100, and 200 ng mL\(^{-1}\)) that had been stored at room temperature for five days. The stability was expressed as a ratio of the results of the incubated controls and the original measured concentrations. The stability of the dried blood spots to freeze-thaw conditions was evaluated by subjecting five control sets (20, 100, and 200 ng mL\(^{-1}\)) to three daily freeze-thaw cycles. Freeze-thaw stability was expressed as a ratio of the observed means versus the respective target concentration.

Post-collection synthesis of POPE and other alcohol biomarkers when specimens have been exposed to or contaminated with ethanol have been reported under a variety of conditions. In the field, it is reasonable to expect that on occasion a specimen will be collected from an individual with a measurable blood alcohol content. An evaluation of this issue consisted of preparing controls fortified with ethanol (316 mg dL\(^{-1}\)). Dried blood spots were prepared before fortification with ethanol as a baseline check, after fortification with ethanol and after incubation at room temperature for 72 hours. Reports have also been published demonstrating post-collection synthesis of alcohol content (BAC) and for specimens stored in the presence of ethanol vapor.

The LOD and LOQ were determined by analyzing a series of fortified controls in triplicate. The LOQ was the lowest point where the mean of the measured concentrations was within 20% of target value and satisfied all identification criteria. The LOD was the lowest triplicate that satisfied all identification criteria without consideration of the measured concentration. The concentrations assayed were 2, 4, 8, 10, and 20 ng mL\(^{-1}\).

Linearity was determined by analyzing a series of fortified negative dried blood spots in quadruplicate. Calibration curves were constructed using analyte/internal standard area response ratios. A weighted (1/\(x\)) least squares linear regression was used to mitigate heteroscedasticity. The concentrations tested were 8, 10, 20, 50, 100, 200, 300, 400, 600, and 800 ng mL\(^{-1}\). Each point was required to be within 15% of target with the exception of the LOQ (8 ng mL\(^{-1}\)) where 20% was allowed.

The potential for carryover was determined by analyzing a known negative control after a control containing 800 ng mL\(^{-1}\). A successful carryover challenge must be less than the determined LOD.

Evaluating 6 negative controls spiked at 500 ng mL\(^{-1}\) with a cocktail of potentially interfering substances (Table 1) assessed the specificity of the assay. The results of the 6 controls must be less than the LOD of the assay. Analyzing 6 LOQ controls fortified at 500 ng mL\(^{-1}\) with a cocktail of potentially interfering compounds challenged the selectivity of the method. All 6 replicates must satisfy the identification criteria and the measured concentrations must be within 20% of target value.

Accuracy and precision were determined by analyzing prepared controls at three different concentrations, replicates of five over four different days. The concentrations investigated were 20, 100, and 200 ng mL\(^{-1}\). The accuracy and precision challenge was considered to be successful if each intra-assay mean and the inter-assay mean were within 15% of target value and the maximum intra- and inter-assay variance must be less than 20%, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Class</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamines</td>
<td>Amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, methamphetamine, ephedrine, pseudoephedrine, phenylpropanolamine, and phenetermine</td>
</tr>
<tr>
<td>Cocaines</td>
<td>Cocaine, cocaethylene, benzoylcegonine, and norcocaine</td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>THC</td>
</tr>
<tr>
<td>Hallucinogens</td>
<td>Phencyclidine, ketamine, norketamine, dextromethorphan, and dextrophan</td>
</tr>
<tr>
<td>Opiates</td>
<td>Codeine, dihydrocodeine, morphine, hydrocodone, hydroxycodeine, oxycodone, oxymorphine, mono-acetylmorphine, buprenorphine, norbuprenorphine, nalbuphine, naltrexone, 6beta-naltrexol, butorphanol, meperidine, normeperidine, pentazocine, tramadol, methadone, 2-ethyliden-1,3-dimethyl-2-piperidinium, fentanyl, norfentanyl, sufentanil, allentanil, propoxyphene, and norpropoxyphene</td>
</tr>
<tr>
<td>Benzodiazipines</td>
<td>Alprazolam, 2-hydroxyalprazolam, diazepam, nordiazepam, oxazepam, midazolam, triazolam, and temazepam</td>
</tr>
<tr>
<td>Barbiturates</td>
<td>Amobarbital, butalbital, pentobarbital, secobarbital, and phenobarbital</td>
</tr>
<tr>
<td>Antidepressants</td>
<td>Fluoxetine, norfluoxetine, amitriptyline, nortriptyline, doxepin, nordoxepin, and sertraline</td>
</tr>
<tr>
<td>Antihistamines</td>
<td>Pheniramine, chlorpheniramine, brompheniramine, doxylamine, and diphenhydramine</td>
</tr>
<tr>
<td>NSAID(^a)</td>
<td>Ibuprofen, naproxen, ketoprofen, and salicylic acid</td>
</tr>
<tr>
<td>Alcohol metabolites</td>
<td>Ethyl glucuronide, ethyl sulfate, ethyl palmitate, ethyl palmitoleyl, ethyl stearate, ethyl oleate, ethyl linolate, ethyl linolenate, and ethyl arachidonate</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidic acid</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Lidocaine, cotinine, hydroxycotinine, caffeine, carisoprodol, meprobamate, methylphenidate, ritalinic acid, zolpidem, zopiclone, and acetaminophen</td>
</tr>
</tbody>
</table>

\(^a\) Non-steroidal anti-inflammatory drug.
fatty acid ethyl esters, a series of ethanol biomarkers, when test specimens were exposed to ethanol vapor. In response to this report, we examined dried blood spots prepared with negative blood, which were then stored at room temperature in an airtight 1000 mL beaker containing an open 10 mL vial of ethanol.

Application of method to prepared dried blood spots

The method was applied to 281 blood specimens that had been previously analyzed in our laboratory and were destined for destruction. POPE concentrations were first determined using whole blood and a slightly modified previously published procedure. Dried blood spots of the 281 specimens were prepared by applying 30 μL of each blood specimen to a Whatman 903 Specimen Collection Paper. The blood spots were allowed to dry for at least 3 hours, according to the instructions of the manufacturer and subjected to the method. Blanks were analyzed between each sample to eliminate carryover concerns. Specimens greater than the upper limit of linearity (800 ng mL⁻¹) were re-evaluated using a single spot.

Results and discussion

Validation results

The parameters and transitions for the mass spectrometry were consistent with a previous report. The precursor ion for the analytes was the deprotonated molecular weight ion m/z 701.5 and m/z 741.7 for POPE and PPro, respectively. The product ion(s) for each compound corresponded to its fatty acid moiety(s), m/z 254 of the palmitic acid and m/z 280 of the oleic acid (Fig. 1). The POPE m/z 701.5 > 255.2 transition was used for quantification. The POPE m/z 701.5 > 281.2 was used as the qualifier. These transitions proved to be clean and stable throughout the validation process. The number of identification points for POPE was 4, satisfying the commonly accepted recommendation of at least 3 identification points. Extracted ion chromatograms of an 8 ng mL⁻¹ LOQ control of POPE are presented in Fig. 2.

The determined LOD for this method was 2.0 ng mL⁻¹. The method allowed for the proper identification of POPE for the 20, 10, 8.0, 4.0 and 2.0 ng mL⁻¹ controls. However, the mean of the measured concentrations of 4.0 and 2.0 controls was outside the required 20% range. The LOQ for this assay was 8.0 ng mL⁻¹. All identification criteria and quantitation criteria for the 8.0 ng mL⁻¹ controls were acceptable with a mean concentration of 7.7 ng mL⁻¹ and %CV of 4.4%.

Linearity of the method was assessed by replicate analysis (n = 4) of negative blood fortified at 8, 10, 20, 50, 100, 200, 300, 400, 600, and 800 ng mL⁻¹. Standard lines were constructed using a 1/x weighted linear regression. The assessment yielded a mean slope of 1.444 ± 5.4 × 10⁻² and an intercept of 1.484 ± 9.155 × 10⁻². The mean coefficient of determination (r²) was 0.9970 ± 0.0023. All points were within 15% of target value.

POPE was not detected (<LOD) in a negative control analyzed immediately following a control fortified 800 ng mL⁻¹ of POPE. The potential for carryover at 800 ng mL⁻¹ of POPE was acceptable.

Negative controls spiked with 500 ng mL⁻¹ of 97 potentially interfering compounds did not exhibit any detectable POPE at or above the reported LOD. The selectivity of the method proved to be adequate by successful analysis of 6 LOQ controls prepared from negative blood that were spiked with 500 ng mL⁻¹ of 97 potentially interfering compounds. The mean of the 6 LOQ controls was 7.94 ng mL⁻¹ and the %CV was 9.1%.

The accuracy and precision of the method proved to be acceptable. The results are listed in Table 2. All intra- and inter-assay accuracy determinations were within 10.8% of target concentration. All intra- and inter-assay precision calculations were less than 8.7%.
The extraction efficiency and matrix effect were determined over 4 concentrations using replicates of 5. Extraction efficiencies ranged from 56.0% to 82.9%. The significant absolute matrix effect was observed with ranges of 40.1% to 47.9%. The results are listed in Tables 3 and 4. Standard lines were constructed using set 3 analyte/internal standard area response ratios. The precision of the standard line slopes was calculated (relative matrix effect) to be 8.8%.

Re-injection of 20, 100 and 200 ng mL\(^{-1}\) controls after incubating 5 days at room temperature did not demonstrate any obvious degradation. The results ranged from 98.5% to 100.7% of the original measured concentrations. The 5 control sets subjected to 3 freeze–thaw cycles proved to be stable. The % target values ranged from 92.7% to 110.4%.

To mimic the conditions in the field when collecting a specimen from an intoxicated donor, five blood specimens were fortified with 316 mg dL\(^{-1}\) of ethanol. They did not demonstrate any detectable POPE when dried blood spots were prepared immediately before and soon after being spiked with the alcohol. However, aliquots of whole blood prepared from the same fortified specimens, after incubating at room temperature for 3 days, demonstrated significant POPE \textit{in vitro} synthesis. The whole blood POPE concentrations ranged from 32 to 292 ng mL\(^{-1}\). All 5 dried blood spots exposed to ethanol vapor for 3 days at room temperature did not demonstrate any detectable POPE.

Caution has been advised when interpreting FAEE, urine ETG, and whole blood POPE for medico-legal issues because of post-collection synthesis. Our experiments demonstrated that dried blood spot specimens contaminated with ethanol (measurable blood alcohol content or exposure to ethanol vapor) do not form this alcohol metabolite once collected. The exact mechanism of inhibition was not determined but it seems logical that the ethanol content evaporated during specimen drying prior to measurable POPE formation. Dried blood spots provide a simple solution to a serious drawback associated with these markers.

**Application of method**

The dried blood spots of 281 specimens were analyzed for POPE. The measured concentrations were compared to results gathered using a previously published whole blood method. A linear

![Fig. 3](image1.png) **Fig. 3** Linear regression comparison of POPE concentrations in authentic blood specimens using whole blood and dried blood spots. The dashed line represents unity.

![Fig. 4](image2.png) **Fig. 4** Bland–Altman plot of POPE comparing whole blood and dried blood spots. Solid line represents the bias of the two methods and the dashed lines enclose a 2 standard deviation range around the bias.
regression analysis (Fig. 3) was performed and showed good correlation \((r = 0.9428)\). A Bland–Altman plot (Fig. 4) was constructed by charting, for each matched pair, the percent difference from the mean of the matched pair versus the mean of the matched pair. The percent difference transformation of the y-axis was chosen over the absolute difference to take into account the increase of standard deviation (s) with concentration. The calculated bias was \(−4.5 \pm 33.8\%\). The calculated bias is depicted as a solid black line with two dashed lines at \(\pm 2s\).

Visual inspection of the Bland–Altman chart indicates an increasing variance with decreasing mean concentration. The commonly expected limit of quantitative agreement for our industry is \(\pm 20\%\). Diminished agreement is most likely due to the greater influence of matrix effects with lower POPE concentrations for both methods.

### Conclusion

A fully validated method was presented for the detection of POPE, an aberrant phospholipid formed in the presence of ethanol, using dried blood spots. This method was applied to 281 specimens that had been analyzed using a previously published method. Comparison of the method to a previously published method using whole blood demonstrated excellent correlation but the agreement needs improvement.

The primary technique in the LCMSMS laboratory for improvement of relative extraction efficiency and relative matrix effect is the use of a stable isotope labeled (SIL) internal standard. At this time, a SIL internal standard for POPE is not commercially available. To date, efforts to acquire a suitable custom synthesized SIL internal standard have been unsuccessful. The current method demonstrated a relative matrix effect of 8.8%, which is greater than the 4% suggested by Matuszewski. The comparison of this method with a previously published whole blood procedure exceeded a desired 20% quantitative agreement. Improvement of the relative matrix effects and relative extraction efficiency for both methods is expected once a SIL internal standard becomes available and consequently will improve the agreement of both of these methods.

Matuszewski indicated that the relative matrix effect must be less than 4% to make the claim that the assay is free of matrix effect. The finding of 8% for our assay must be considered when interpreting results in the real world. The use of this current assay to predict consumption history on an individual basis may be confounded by the relative matrix effect. However, at this time, the intended use of this assay is simple detection primarily for relapse detection and therapy evaluation. Efforts to improve this assay are on-going in our laboratory.

The detection of POPE in blood is gaining recognition as a reliable, specific long-term direct biomarker of ethanol consumption. Using dried blood spots for POPE testing allows for collection in a nonclinical setting without the services of highly trained collectors. The collection is simple, less invasive, and virtually painless making it an ideal test matrix for broad epidemiological studies of alcohol intake. This method will be utilized as an objective marker in markets where detection of early relapse and abstinence verification is critical as opposed to precise estimation of ethanol intake.

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