

The Detection of 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol and Ethyl Glucuronide in Human Umbilical Cord*

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ABSTRACT

In utero exposure to ethanol continues to be a significant public health issue and neonatal healthcare professionals are in need of objective means to identify exposed newborns. The aim of this study was to fully validate two methods for the detection of two direct alcohol biomarkers, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (POPE) and ethyl glucuronide (EtG), in umbilical cord and apply the assays to a group of authentic specimens. The limits of detections were 2 and 1 ng/g for POPE and ETG and the limits of quantitation were 4 and 3 ng/g, respectively. Inter and intra-day precision and accuracy measurements were within 15%. The assays were applied to 308 authentic specimens where we detected POPE in five (1.6%) specimens and EtG in twelve (3.9%) specimens. The mean concentrations were $11.4 \text{ ng/g} \pm 9.4 \text{ ng/g}$ and $127.2 \pm 227.7 \text{ ng/g}$ for POPE and EtG, respectively. This study suggested that umbilical cord was a suitable specimen type for the identification of newborns exposed to ethanol in the womb and the prevalence of POPE and EtG detected in umbilical cord were consistent with the prevalence of self-reported binge drinking reported by the National Birth Defect Prevention Study (NBDPS) and Behavioral Risk Factor Surveillance System (BRFSS). Further studies are required to fully describe the association between the observed concentrations of POPE and EtG in umbilical cord to the level of maternal consumption of ethanol.

Keywords: Phosphatidylethanol; Ethyl Glucuronide; Umbilical Cord; Ethanol; Fetus; LC-MS/MS; Prenatal Exposure; Fetal Alcohol Spectrum Disorders; FASD

1. Introduction

The reduction of harmful *in utero* exposure of a developing fetus to alcohol was noted as one objective of Healthy People 2020, a national health improvement and disease prevention campaign [1]. This goal implied the ability to identify alcohol exposed newborns. However, in the absence of gross physical malformation and an accurate maternal drinking history, exact and consistent identification has been a challenge [2,3].

The most prevalent screening tool to identify newborns exposed to alcohol *in utero* has been the self-report questionnaire. The National Birth Defects Prevention Study, a large CDC-sponsored anonymous survey, observed that 6.5% of mothers self-reported drinking alcohol during the third trimester with 0.5% binge drinking in the third trimester [4]. Recent Behavioral Risk Factor Sur-

veillance System (BRFSS) data, a large random-digit-dialed telephone survey, revealed that 7.6% of pregnant women drank during pregnancy and 1.4% self-reported binge drinking [5]. Both studies conceded probable underestimation because the required maternal cooperation for an accurate self-report may be lacking due to self incrimination, social stigma, misclassification and recall bias [4-8]. A reliable and objective alcohol biomarker to identify *in utero* exposure to ethanol would be beneficial to facilitate intervention to the newborn and to provide objective outcome measures for preventive intervention researchers [9].

For over a decade, the detection of fatty acid ethyl esters (FAEE), a series of non-oxidative direct alcohol biomarkers, in meconium has been performed routinely and has been considered the gold standard method for the identification of newborns exposed to ethanol *in utero* [10-15]. The primary drawback of meconium testing has been the high percentage of specimens (9% to 27%) that are unavailable for testing due to passage *in utero*, inadvertent specimen destruction, and inadequate volume

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[16-22]. Moore *et al.* [10] demonstrated that FAEE in meconium were susceptible to photo and thermal degradation which complicates collection, shipping, and storage strategies. A truly universal and robust specimen type would be helpful to caregivers to screen for *in utero* exposure to ethanol.

Umbilical cord (UC) has been suggested as an alternative specimen type for newborn toxicology [23,24]. The detection of a drug of abuse, specifically benzoylecgonine (cocaine metabolite), in UC was first reported in 1993 [25]. The detection of benzoylecgonine in UC tissue has been used as forensic evidence of maternal drug use [26] and that interpretation was upheld on appeal to the South Carolina Supreme Court in 2008 [27]. Reports of UC for the detection of amphetamines [28], opiates [29-31], caffeine [32] and tobacco metabolites [33,34] have been published in recent years.

Phosphatidylethanol (PEth), a group of abnormal phospholipids, are formed in the presence of ethanol, phosphatidylcholine, and phospholipase D [35]. They are considered direct alcohol biomarkers because of the incorporation of the original ethanol moiety in the final product. Structurally, PEth consists of a phosphoethanol head connected to 2 fatty acid moieties which determines the specific isomer. The palmitoyl/oleoyl species (POPE) was shown as the most prevalent isomer of PEth (40% of total PEth) [36,37]. Once formed, PEth incorporates into the phospholipid membranes of blood and tissue cells and decomposes with a half-life of 4 - 5 days, which allows for a wide window of detection after excessive alcohol consumption [36,38].

Ethyl glucuronide (EtG) is a product of the conjugation of ethanol with activated glucuronic acid and is, therefore, a direct alcohol biomarker. Once formed, EtG is eliminated with a half life of 2 - 3 hours with a reported detection window of up to 80 hours following excessive consumption [39]. The detection of EtG has been reported in a number of newborn specimen types including meconium, placenta, and fetal remains [15, 40-44]. A report using umbilical cord tissue to detect a direct alcohol biomarker is lacking in the literature at this time.

Following every birth, a sufficient quantity of UC, waste material genetically belonging to the neonate [45-47], is available for diagnostic analysis. We hypothesize that a newborn's UC may be used for the detection of the direct alcohol biomarkers, POPE and EtG. This report consists of two elements. First, we intend to present fully validated LC-MS/MS methods for the analyses of POPE and EtG in UC. The second element used these methods to analyze UC specimens received in our laboratory and compared the outcomes to the most recent estimates of excessive maternal drinking.

2. Experimental

2.1. Ethics Statement

The UC specimens used in this study were referred to our laboratory for routine analysis. POPE and EtG analyses were performed on de-identified aliquots of specimens remaining after the original intended analysis. These aliquots were considered to be waste and did not require an ethics review.

2.2. Subjects

Over a period of 7 weeks, duplicate 1 gram aliquots of UC were harvested from specimens received into the laboratory provided that a sufficient amount of specimen was received. The de-identified aliquot was transferred to a 50 mL polypropylene conical tube and stored at -20°C until time for analysis.

2.3. Chemicals, Reagents and Materials

The internal standard, 1-palmitoyl (d_{31})-2-oleoyl-*sn*-glycero-3-phosphoethanol (POPE- d_{31}), was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The PEth of interest, POPE, was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). EtG and EtG- d_5 was purchased from Cerilliant (Round Rock, TX, USA) as 1 mg/mL ampoules. All reagents (ACS grade) and all solvents (HPLC grade) were purchased from Thermo-Fisher (Hanover Park, IL, USA). Anion exchange solid phase extraction columns (aminopropyl, CUNAX12Z, 200 mg bed, 10 mL cartridge and Quaternary Amine with Chloride Counter Ion, CUQAX12Z, 200 mg bed, 10 mL cartridge) were purchased from United Chemical Technologies (Bristol, PA, USA).

2.4. Preparation of Calibration Standards and Quality Control Samples-POPE

Stock solutions of POPE and POPE- d_{31} were prepared in chloroform at a concentration of 100 $\mu\text{g}/\text{mL}$. Using different lots of POPE stock solutions, working solutions (400 ng/mL) were prepared by further dilution with mobile phase A (20% 2 mM ammonium acetate: 58% acetonitrile: 22% isopropanol). An internal standard working solution (POPE- d_{31} , 800 ng/mL) was prepared by appropriate dilution with mobile phase A. Using different lots of working solutions, a calibrator (10 ng/g) and a set of controls (0, 4, 12.5, and 80 ng/g) were prepared by spiking 1 gram of negative UC in a 50 mL polypropylene conical bottom tube with an appropriate amount of POPE working solution.

2.5. Preparation of Calibration Standards and Quality Control Samples-EtG

Stock solutions of EtG and EtG- d_5 were prepared in

methanol at a concentration of 100 µg/mL. Using different lots of EtG stock solutions, working solutions (100 ng/mL) were prepared by further dilution with methanol. An internal standard working solution (EtG-*d*₅, 200 ng/mL) was prepared by appropriate dilution with methanol. Using different lots of working solutions, a calibrator (10 ng/g) and a set of controls (0, 2, 6, 15, and 40 ng/g) were prepared by spiking 1 gram of negative UC in a 5 mL polypropylene conical bottom tube with an appropriate amount of EtG working solution.

2.6. Specimen Preparation-POPE

The specimens were prepared by accurately transferring 1.0 gram of UC to 50 mL polypropylene conical bottom tubes. To each specimen, calibrator or control, 50 mL of POPE-*d*₃₁ internal standard working solution was added (40 ng/g) to each tube. To each tube, 5.5 mL of hexane/isopropanol (3:2) was added. Each calibrator, control and specimen was homogenized using a PRO Scientific 20 mm × 200 mm PRO250 homogenizer (Oxford, CT, USA). The tubes were centrifuged at approximately 650 *xg* for approximately 5 min. The extract was loaded onto the solid phase extraction columns that had been conditioned with 2 mL of hexane and allowed to flow through under the force of gravity. The eluates were captured in clean labeled 13 × 100 mm glass tubes and evaporated under a stream of nitrogen at 40°C. The residue was reconstituted in 200 L of mobile phase A (20:58:22; 2 mM ammonium acetate/acetonitrile/isopropanol) and analyzed by LC-MS/MS.

2.7. Specimen Preparation-EtG

The specimens were prepared by accurately transferring 1.0 gram of UC to 5 mL polypropylene conical bottom tubes. To each specimen, calibrator or control 50 mL of EtG-*d*₅ internal standard working solution was added (20 ng/g). To each tube, 3 mL of acetonitrile and 3 stainless steel wood screws were added. The tubes were capped and placed in the Bullet Blender® (Next Advance, Averill Park, NY, USA) at speed setting 7 for approximately 5 min. The tubes were centrifuged at approximately 650 *xg* for approximately 5 min. The supernatants were decanted to clean labeled 13 × 100 mm glass tubes and evaporated under a stream of nitrogen at 40°C. The residues were reconstituted in 3 mL of deionized water and loaded onto quaternary amine anion exchange solid phase extraction columns conditioned with 2 mL of methanol and 2 mL of deionized water. The extracts flowed through under the force of gravity without any assistance. The cartridges were rinsed with 2 mL of deionized water and 2 mL of methanol and eluted with 2 mL of 2% formic acid in methanol. The eluates were captured in clean labeled 13 × 100 mm glass tubes and evaporated under a stream of

nitrogen at 40°C. The residues were reconstituted in 1 mL of deionized water and transferred to labeled 2 mL ALS vials for LC-MS/MS analysis.

2.8. LCMSMS Conditions-POPE

The specimens were analyzed using an Agilent Technologies 1200 system that consisted of a G1367D autosampler, a G1379B degasser, G1312B binary pump, and a G1310 isocratic pump (Wilmington, DE, USA). Separation was achieved using an Agilent Poroshell 120 EC-C8 (50 mm × 2.1 mm, 2.7 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, 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 3500V, the nozzle voltage set at 500V and the desolvation gas (nitrogen) was heated to 300°C with a flow of 5 l/min. The sheath gas (nitrogen) was heated to 250°C and delivered at 11 l/min (Wilmington, DE, USA). The internal standard (POPE-*d*₃₁) was monitored using the *m/z* 733.0 > 281.4 (quantification ion; Frag = 160; CE = 38) and *m/z* 733.0 > 286.6 (qualifying ion; Frag = 160; CE = 30) transitions. The *m/z* 702.0 > 281.3 (quantification ion; Frag = 165; CE = 34) and *m/z* 702.0 > 255.3 (qualifying ion; Frag = 165; CE = 34) transitions were used to monitor POPE where Frag is the Fragmentation Voltage (V) and CE is the Collision Energy (V). All data were processed using MassHunter B.02.01 (Wilmington, DE, USA).

2.9. LCMSMS Conditions-EtG

The specimens were analyzed using an Agilent Technologies 1200 system as described above. Separation was achieved using a Synergi Polar RP (50 mm × 2.0 mm, 2.5 µm particle size) C-18 column (Phenomenex, Torrance, CA, USA). The column was held at 30°C in a G1316B Thermostatted Column Compartment (Wilmington, DE, USA). The solvent system was a gradient that consisted of A (deionized water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), using a flow rate of 0.100 mL/min. The solvent program held B at 1% from 0.0 min to 4.0 min. Solvent B was increased to 99% between 4.0 min and 4.1 min and held until 5.1 min. Solvent B was decreased to 1% between 5.1 min and 5.2 min and held at 1% until 10.0 min. The detector

was an AB Sciex Triple Quad™ 5500 tandem mass spectrometer using electro-spray ionization (ESI) in the negative mode (Foster City, CA, USA). The ion spray voltage was -4200 V, the source temperature was 650°C . The curtain gas and collision gas was nitrogen held at 30 psi and 5 psi, respectively. The internal standard (ETG $-d_5$) was monitored using the m/z 226.1 $>$ 75.0 (quantification ion; DP = 65; CE = 22; CXP = 13) transition and the m/z 226.1 $>$ 85.0 (qualifying ion; DP = 65; CE = 22; CXP = 13) transition. The m/z 221.1 $>$ 75.0 (quantification ion; DP = 75; CE = 21; CXP = 22) and m/z 221.1 $>$ 85.0 (qualifying ion; DP = 44; CE = 23; CXP = 22) transitions were used to monitor ETG, where DP is the declustering potential (V), CE is the collision energy (V), and CXP is the collision cell exit potential (V). The dwell time for monitoring each transition was 400 msec. All data were processed using Analyst 1.5.1 (Foster City, CA, USA).

2.10. 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.

2.11. Method Validation

The method was validated according to the recommendations of commonly accepted guidelines [48-51]. The following parameters were evaluated for each assay: limit of detection (LOD), limit of quantitation (LOQ), linear range, carryover potential, selectivity, specificity, bias, imprecision, 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 alcohol content (BAC) and for specimens stored in the presence of ethanol vapor.

The LOD and LOQ for each assay 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 1, 2, 3, 4, 5, 8, and 10 ng/g.

Linearity for each assay was determined by analyzing

a series of 5 fortified negative UC between the concentrations of 4 and 200 ng/g in quadruplicate. Calibration curves were constructed using analyte/internal standard area response ratios. A weighted ($1/x$) least squared linear regression was used to mitigate heteroscedasticity. The means and standard deviations of the calibration curve slopes and intercepts were calculated.

The potential for carryover for each assay was determined by analyzing a known negative control after a control containing 800 ng/g. A successful carryover challenge must be less the determined LOD.

Evaluating 6 negative controls spiked with a cocktail of potentially interfering substances (**Table 1**) assessed the specificity of each assay. The results of the 6 controls must be less than the LOD of its respective assay. Analyzing 6 LOQ controls fortified 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.

Bias and imprecision of each assay were determined by analyzing prepared controls at three different concentrations, replicates of five over four different days. The concentrations investigated were 5, 40, and 75 ng/g for EtG and 10, 50, and 100 ng/g for POPE. The bias and imprecision 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.

The extraction efficiency and matrix effect were determined using procedures defined by Matuszewski [50,51]. To determine the matrix effect and extraction efficiency, three sets of controls were prepared over three concentrations with five replicates each. The concentrations analyzed for each assay were 10, 100 and 200 ng/g for POPE and 5, 100, and 200 ng/g for EtG.

The first set was unextracted controls reconstituted in mobile phase A. The second set was negative UC extracts fortified with POPE or EtG after being subjected to its respective extraction procedure. The third set was negative UC controls obtained from five different sources fortified with POPE or EtG that were subjected to the extraction procedures. The extraction efficiency for each analyte was expressed as the ratio of the average peak area in set 3 to set 2. The matrix effect for each analyte was defined as the ratio of the mean peak area of set 2 to set 1. The relative matrix effect was defined as the coefficient of variation (%CV) of the standard line slopes constructed from negative UC controls obtained from five different biological sources fortified with POPE or EtG that were subjected to the extraction procedure. Five replicates of five concentrations were used that ranged from 4 to 200 ng/g. A relative matrix effect of less than 4.5% was considered acceptable [51].

Table 1. List of potentially interfering compounds used to evaluate the selectivity and specificity of PEth and EtG in UC.

Drug Class	Compounds
Amphetamines	amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, ephedrine, pseudoephedrine, phenylpropanolamine, phentermine,
Cocaines	cocaine, cocaethylene, benzoylecgonine, norcocaine
Cannabinoids	THC
Hallucinogens	phencyclidine, ketamine, norketamine, dextromethorphan, dextrorphan
Opiates	codeine, dihydrocodeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone, mono-acetylmorphine, buprenorphine, norbuprenorphine, nalbuphine, naltrexone, 6-naltrexol, butorphanol, meperidine, normeperidine, pentazocine, tramadol, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, fentanyl, norfentanyl, sufentanil, alfentanil, propoxyphene, norpropoxyphene,
Benzodiazepines	alprazolam, α -hydroxyalprazolam, diazepam, nordiazepam, oxazepam, midazolam, triazolam
Barbiturates	amobarbital, butalbital, pentobarbital, secobarbital, phenobarbital
Anitidepressants	fluoxetine, norfluoxetine, amitriptyline, nortriptyline, doxepin, nordoxepin, sertraline
Antihistamines	pheniramine, chlorpheniramine, brompheniramine, doxylamine, diphenhydramine
NSAID ^a	ibuprofen, naproxen, ketoprofen, salicylic acid
Miscellaneous	lidocaine, cotinine, hydroxycotinine, caffeine, carisoprodol, meprobamate, methylphenidate, ritalinic acid, zolpidem, zopiclone, acetaminophen

^aNon-steroidal Anti-inflammatory drug.

The stability of prepared extracts was assessed by the re-analysis of a control set from the bias and imprecision experiment 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 to freeze-thaw conditions were evaluated by subjecting a control set from the precision and accuracy experiment 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 syntheses of EtG, POPE, and other alcohol biomarkers in specimens exposed to or contaminated with ethanol have been reported under a variety of

conditions [52-55]. In the field, it is reasonable to expect that a UC specimen may be exposed to ethanol either intentionally or unintentionally. To evaluate the potential for *in vitro* formation of POPE and EtG in UC, we examined 2 aliquots from 3 negative UC specimens. One aliquot from each specimen was stored for 2 days at room temperature in an airtight 1000 mL beaker containing an open 5 mL vial of ethanol and the second aliquot was stored at room temperature without being exposed to ethanol vapor.

To determine if these biomarkers are present at consistent levels throughout the UC, a longitudinal study was performed on a positive and negative specimen. Six aliquots were taken at equally spaced intervals along a 6 inch section of UC. The mean, standard deviation, and %CV were calculated for each specimen.

2.12. Application of Method to Authentic Specimens

The methods were applied to 308 de-identified UC that were received by our laboratory for routine toxicological analysis.

2.13. Statistical Analysis

Statistical analysis was performed using IBM[®] SPSS[®] Statistic Version 19.0.0. Pearson correlation was used to evaluate the association between POPE and EtG observed in authentic UC. A probability of $P \leq 0.01$ was considered to be significant.

3. Results

3.1. Validation Results

The parameters and transitions for the mass spectrometry were consistent with previous reports [43,56,57]. The precursor ions for the PEth analytes were the deprotonated molecular weight ions m/z 702.0 and m/z 733.0 for POPE and POPE- d_{31} , respectively [37,56,57]. The product ion(s) for each POPE corresponded to its fatty acid moiety(s), m/z 255 of the palmitic acid, m/z 281 of the oleic acid, and m/z 281 of the per-deuterated palmitic acid (**Figure 1**) [37,56,57]. The precursor ions for the EtG and EtG- d_5 were the deprotonated molecular weight ions m/z 221.0 and m/z 226.1 for EtG and EtG- d_5 , respectively (**Figure 2**). The product ion used for quantitation (m/z 75) has been proposed to be the 2-hydroxyethanoate ion or the 2-hydroxy-1-propoxide ion and the qualifying ion for EtG (m/z 85) has been proposed to be the 2-hydroxy-3-buten-1-oxide anion [58]. Both fragments are remnants of a complex ring opening and multiple step fragmentation [58]. The number of identification points for both compounds was 4, satisfying the com-

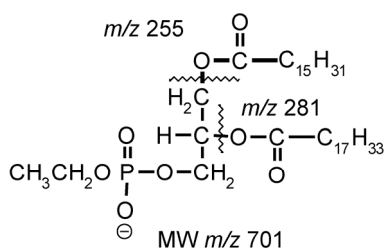


Figure 1. Structure and fragmentation pattern of POPE.

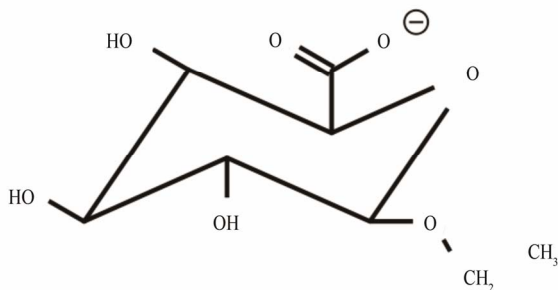


Figure 2. Structure of EtG.

monly accepted recommendation of at least 3 identification points [49]. Extracted ion chromatograms of a LOQ control for POPE and EtG are presented in **Figures 3** and **4**.

The determined LOD and LOQ for POPE were 2.0 and 4.0 ng/mL, respectively. The determined LOD and LOQ for EtG were 1.0 and 3.0 ng/mL, respectively. The specificity of each assay was considered acceptable because POPE and EtG were not detected (<LOD) in a set of negative controls that were fortified with a cocktail of potentially interfering substances. The selectivity of each assay was considered to be acceptable due to the satisfactory identification and quantitation of a set of LOQ controls spiked with potentially interfering compounds. POPE or EtG was not detected in negative controls analyzed following controls fortified with 800 ng/g of POPE or EtG.

The relative matrix effects were 3.4% and 4.3% for POPE and EtG, respectively. The accuracy, precision and linearity calculations are presented in **Table 2**. The absolute matrix effect and extraction efficiency results are posted in **Table 3**. The stability data are listed in **Table 4**. All bias determinations of the validation were within 14.7% of target concentration. All imprecision calculations were less than 7.6%.

The 3 ethanol-vapor-exposed aliquots formed POPE and EtG *in vitro* after standing at room temperature for 2 days. POPE and EtG were not detected in the 3 non-exposed segments. The exposed aliquots produced between 208 and 1029 ng/g of POPE and between 11 and 201 ng/g of EtG. The negative specimens were negative

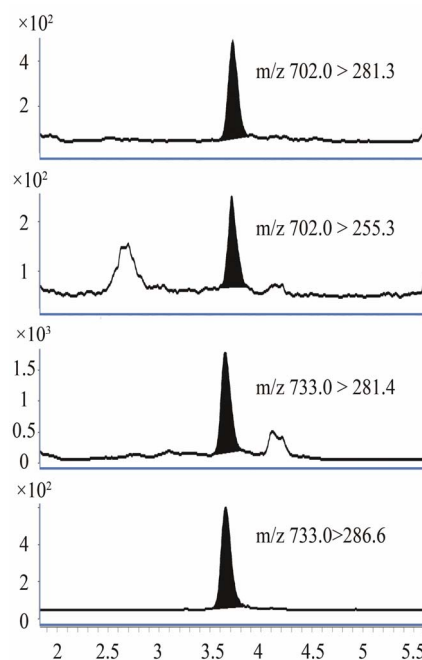


Figure 3. Selected ion chromatogram of POPE and POPE-*d*₃₁ LOQ control (4 ng/g).

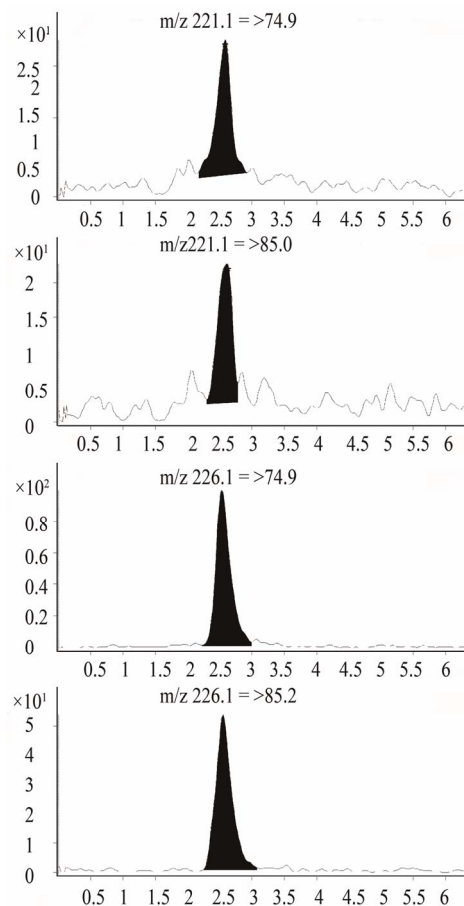


Figure 4. Selected ion chromatogram of EtG and EtG-*d*₅ LOQ control.

Table 2. Precision, accuracy, and linearity of methods for the detection of POPE and EtG in UC.

Compound		Intra-assay (n = 5)		Inter-assay (n = 20)	
	Target concentration (ng/g)	Accuracy (%)	Precision (%CV)	Accuracy (%)	Precision (%CV)
POPE	10	95.5 - 103.2	2.5 - 4.5	99.6	4.2
	50	96.6 - 108.2	3.0 - 6.6	104.4	6.1
	100	96.6 - 107.1	1.6 - 3.7	101.8	4.9
Calibration curve (n = 4) slope = 0.0268 ± 0.008; intercept = -0.0321 ± 0.019; r ² = 0.9992 ± 0.0004					
EtG	5	103.2 - 113.9	3.7 - 7.6	108.7	7.5
	40	85.3 - 95.4	2.9 - 4.0	87.9	7.4
	75	85.6 - 93.6	1.9 - 5.7	87.6	7.6
Calibration curve (n = 4) slope 0.0569 ± 0.002; intercept = 0.0415 ± 0.017; r ² = 0.9992 ± 0.0009					

Table 3. Matrix effect and extraction efficiency data for the detection of POPE and EtG in UC.

Compound	Target (ng/g)	Matrix Effect Absolute (%)	Extraction Efficiency (%)
POPE	10	22.8	58.3
	100	82.1	14.2
	200	84.4	11.5
POPE-d ₃₁	10	21.9	59.8
	100	91.1	13.9
	200	91.7	13.4
EtG	5	83.0	41.1
	100	84.5	42.0
	200	80.2	45.8
EtG-d ₅	5	81.1	39.4
	100	86.4	46.1
	200	78.3	47.2

Table 4. Stability data for POPE and EtG in UC.

Compound	Target (ng/g)	Extracts 4 days room temperature (% Original Result)	3 Freeze/thaw cycles
POPE	10	100.5	95.5
	50	90.0	106.2
	100	87.0	107.1
	5	98.2	106.2
EtG	40	87.7	84.4
	75	94.3	84.8

throughout the entire length of specimen tested. The longitudinal study for the positive specimen demonstrated that POPE (719 ± 273 ng/g; %CV = 38%) and EtG (2742 ± 85.9 ng/g; %CV = 3%) were consistently found along the length of UC.

3.2. Application of Method

A group of 308 UC specimens that had been received by our laboratory were tested using the newly validated methods. Two hundred and ninety five (295) specimens did not contain detectable amounts of POPE or EtG. Four specimens contained both POPE and EtG. Eight specimens contained EtG and no detectable POPE. One specimen that contained POPE did not contain a detectable amount of EtG.

Five (1.6%) specimens contained a detectable amount of POPE. The POPE mean concentration was 11.4 ng/g ± 9.4 ng/g and the median was 11.0 ng/g. Twelve (3.9%) specimens contained a detectable amount of EtG. The EtG mean concentration was 127.2 ng/g ± 227.7 ng/g and the median was 21.0 ng/g. The Pearson product-moment correlation coefficient (*r*) was 0.576 (*P* = 0.07, *n* = 13). Results of the 13 specimens that contained either POPE or EtG are charted in **Figure 5**.

4. Discussion

We have presented fully validated assays for the detection of two direct alcohol biomarkers, POPE and EtG, in human UC. In addition, we have analyzed 308 authentic UC that had been received into our laboratory for routine analysis for the presence of POPE and EtG. The positivity rates for POPE and EtG in the authentic specimen survey were 1.6% and 3.9%, respectively. When detected, the mean concentration of POPE was 11.4 ng/g ± 9.4 ng/g and the mean concentration of EtG was 127.2 ± 227.7

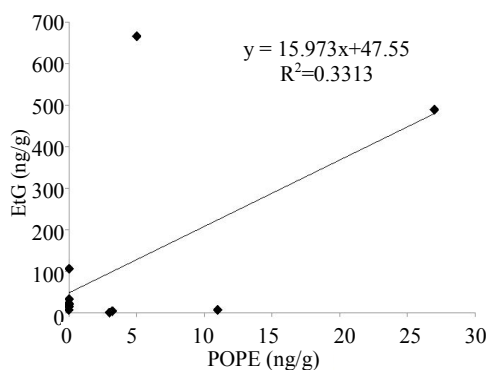


Figure 5. Comparison of results for authentic UC specimens with detectable levels of POPE or EtG.

ng/g. The measured concentrations of POPE and EtG were positively associated ($r = 0.5174$) but the association was insignificant ($P = 0.07$).

POPE was previously reported in 10 types of post-mortem tissue (kidney, lung, spleen, liver, heart, skeletal muscle, small intestine, fat, cerebellum, and brain cortex) from known alcoholics [55]. The measured concentrations of POPE in these tissues were several orders of magnitude higher (9.8 g/g to 937 g/g) than those found in our UC (3 ng/g to 27 ng/g). The elevated levels of the autopsy tissues were proposed to be due in part to *in vitro* synthesis from the significant blood alcohol content of the decedents at the time of death (121 mg/dL to 364 mg/dL) and subsequent freezing of the harvested tissue which further concentrates the ethanol in the tissues [55]. In our study, UC presented a unique opportunity to evaluate the presence of POPE in human tissue other than post-mortem analysis.

The detection of EtG in post-mortem tissues has been suggested as a useful tool to gain insight into a decedent's alcohol history [59]. Once again, EtG levels much higher than our observations were reported presumably influenced by elevated blood alcohol concentrations (106 mg/dL to 183 mg/dL) at the time of death or formed from ethanol due to putrefaction. Morini *et al.* [43] reported the presence of EtG in placenta and the fetal remains of pregnancies that had been voluntarily terminated in the 12th week. The fetal study reported EtG concentrations between 78 ng/g and 1299 ng/g, which were consistent with the levels we observed in our UC survey (4 ng/g to 666 ng/g).

The measured concentrations of EtG in UC in our study (4 ng/g to 666 ng/g; mean 127 ng/g) were found to be similar to concentrations found in recent meconium studies. Bakdash *et al.* [15] reported concentrations between 10 ng/g and 10,230 ng/g (mean 601 ng/g) in a study originating from Erlangen, Germany. Morini *et al.* [41,42] and Pichini *et al.* [44] reported concentrations between 6.9 ng/g and 1443 ng/g from five cities in Italy

and Barcelona, Spain.

The BRFSS reported that 7.4% of women self-reported the use of alcohol during their pregnancy [5]. A very similar prevalence of self-reported drinking specifically during the 3rd trimester (6.5%) was reported by the NBDPS [4]. The BRFSS and NBDPS further reported that 1.4% self-reported binge drinking at some point during the pregnancy and 0.5% self-reported binge drinking during the 3rd trimester. We understand that these findings are underestimated due to obvious limitations of using self-report but provide context for evaluating the prevalence of direct alcohol biomarkers in newborn tissue. The prevalence of POPE and EtG in UC at 1.6% and 3.9%, respectively, may be more consistent with those reporting binge drinking and therefore a more risky behavior.

Our study found that detectable levels of POPE and EtG may be formed *in vitro* by exposure to ethanol vapor. Historically, caution has been advised when interpreting FAEE, urine ETG, and whole blood POPE for medicolegal issues due to post-collection synthesis. That caution applies to these analyses as well. Therefore, it is very important for the collection staff to be aware of this observation and ensure that ethanol containing products are not used on or near the specimen during the collection process.

A limitation of this study was the absence of experimentally determined pharmacokinetics of POPE and EtG in UC, due to obvious ethical concerns. A second limitation of this study was the lack of accurate detailed self-report data concerning maternal consumption of ethanol. Accurate pharmacokinetic and self-report data would have provided insight to the correlation, clinical sensitivity and clinical specificity of these two assays in relation to risky alcohol behavior. Another limitation of this study was that due to productivity concerns in an operational reference laboratory, the two assays were developed at different times on different analytical platforms and the target concentrations chosen for each validation study were not identical.

5. Conclusion

This study provides evidence that umbilical cord tissue is a suitable specimen type to identify *in utero* exposure to ethanol. Umbilical cord tissue is an ideal specimen type for newborn screening programs and large scale epidemiological studies because, when compared to other newborn toxicology specimen types, it is truly a universal specimen and very simple to collect. Recently, detection of the direct alcohol biomarkers, POPE and EtG, have been gaining popularity to monitor risky alcohol drinking behavior in areas such as professional health programs, substance abuse treatment evaluation, and chro-

nic disease management. These assays provide another tool for the neonatal health professional to identify candidates in need of further evaluation.

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