DETERMINING THE ROLE OF PHOSPHOLIPASE-D IN THE CHARACTERIZATION OF THE PHARMACOKINETICS OF PHOSPHATIDYLETHANOL

by

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ABSTRACT

DETERMINING THE ROLE OF PHOSPHOLIPASE-D IN THE CHARACTERIZATION OF THE PHARMACOKINETICS OF PHOSPHATIDYLETHANOL

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Background. The purpose of this study was to determine the role of phospholipase-D (PLD) in the characterization of the pharmacokinetics of phosphatidylethanol(PEth) taken from whole blood samples from participants in a human clinical laboratory study after consumption of ethanol. **Methods.** In the study 27 participants received 0.4g/kg ethanol and 27 participants received 0.8g/kg ethanol during a 15 minute period. Blood samples were collected prior to ethanol consumption and throughout a 6 hour period, as well as every 3 days for the next 2 weeks, post-ethanol consumption. Two PEth homologues were quantified in blood samples by HPLC/MS/MS and data was reported for each homologue as well as combined totals (combined PEth). PLD activity was measured using 490µl of baseline blood samples and 10µl of ethanol in-vitro every hour over a 6 hour period. Breath ethanol concentrations (BrAC) were measured concurrently with each blood draw and transdermal alcohol concentrations (TAC) were measured throughout the 22 day study to ensure abstinence 7 days prior to the study as well as the 14 day period after ethanol consumption. **Results. (**1) There was proportional increase and decrease in combined PEth levels in all participants of both, 0.4 and 0.8g/kg, ethanol dosage groups; (2) the rates of formation and elimination of PEth 16:0/18:2 were higher than those of PEth 16:0/18:1 after ethanol administration and during the subsequent 14 day period of

abstinence; (3) there was no correlation of PLD activity and PEth levels in-vitro. **Conclusion.** The examination of the pharmacokinetics of PEth through this study shows that there are other factors besides PLD activity affecting the rate of synthesis and elimination. In addition, measurement of the two homologues of PEth provides further evidence into the sensitivity of this relatively new alcohol biomarker.

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CHAPTER 1: INTRODUCTION

1.1 Background

Alcohol is a psychoactive substance with dependence-producing properties (WHO, 2015). It is a substance that can lead to physical and mental health problems when consumed in excess quantities (Rehm et al., 2011). Excessive use of alcohol ranks among the top five risk factors for disease, disability, and death throughout the world (WHO, 2011a; Lim et al., 2012). It is associated with a risk of health problems such as alcohol dependence, liver cirrhosis, cancers, fetal alcohol spectrum syndrome, hypertension, depression, hemorrhagic stroke, gastrointestinal bleeding, and unintentional injuries. Excessive alcohol use is also associated with behavioral issues such as violence, criminal acts, property damage, risky sexual behavior, and lost worker productivity (Rehm et al., 2003). The net effect of harmful use of alcohol is approximately 3.3 million deaths each year which accounts for approximately 5.9% of all deaths worldwide (WHO, 2011). In the United States alone, excessive alcohol consumption is the third leading preventable cause of death and accounts for over 79,000 deaths annually. Excessive alcohol use costs the United States about $185 billion each year in health care, criminal justice expenses, and lost productivity (CDC, 2011).

As a consequence, interest has risen in how the problematic consumption of alcohol can be detected and reduced in certain populations. Negative health and societal consequences highlight the importance of developing more effective diagnostic modalities for early detection of hazardous drinking (Niemela, 2007). The investigation of suspected alcohol dependence or abuse by studying symptoms, medical history, self-report forms, questionnaires, and alcohol biomarkers is important for the health of patients and also in forensic settings, medical practice, and treatment of alcoholism (Viel et al., 2012).

Over the past few decades, interest in using alcohol biomarkers has grown because it is a biological estimate of how much alcohol has been consumed. In the current study, phosphatidylethanol (PEth), a relatively new alcohol biomarker in whole blood, is being studied to characterize its pharmacokinetics by understanding how phospholipase-D might have an effect on PEth levels.

1.2 Alcohol Biomarkers

The direct measurement of alcohol through multiple bodily fluids, including blood, saliva, urine, sweat, and expired air is often the most commonly utilized measure for determining recent alcohol exposure (Swift, 2003). However, there exist a few disadvantages to the direct measurement of alcohol for determination of alcohol consumption. Its principal disadvantage is that ethanol has a short half-life in the body of 2 to 3 hours, so it requires frequent testing to determine exposure. Another disadvantage is that alcohol concentrations can vary in different parts of the body depending on the tissue tested (Jones, 1990). Because almost all the alcohol that enters the body is further metabolized, alcohol metabolites that persist in the body for hours to days can determine alcohol consumption (Abudu, 2008).

Alcohol biomarkers, both direct and indirect, are formed endogenously in the blood after ethanol consumption and are proof of alcohol intake. Ingested ethanol is removed from the body through oxidative metabolism (92-95%), nonoxidative metabolism (1%), or excreted unchanged in breath, sweat, and urine (5-8%) (Karch, 2006). In the oxidative pathway, ethanol is oxidized in the liver to acetylaldehyde and acetic acid. Initially, ethanol is dehydrated by the enzyme alcohol dehydrogenase to acetylaldehyde. Then, acetaldehyde is oxidized by the enzyme aldehyde dehydrogenase to acetate (Janda et al., 2001). In the nonoxidative pathway, conjugation reactions are catalyzed by enzymes that include glucuronyltransferases, sulfotransferases, phospholipases, and acyl transferases/esterases. The products of non-oxidative metabolism

include ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanol (PEth), and fatty acid ethyl esters (FAEE). These are described as direct biomarkers. Indirect biomarkers, which are toxic by-products of ethanol consumption, include serum alanine aminotransferase, serum aspartate aminotransferase, serum/plasma carbohydrate deficient transferrin (CDT), mean corpuscular volume (MCV), and serum gamma-glutamyltransferase (GGT) (Wurst et al., 2005).

1.2.1 Indirect and Direct Markers

Indirect alcohol markers suggest heavy alcohol consumption by detecting the toxic effects that alcohol may have on organ systems or body chemistry (SAMHSA, 2006). Included in this class are the blood-based measures of gamma glutamyl transferase (GGT), aspartate amino transferase (AST), alanine amino transferase (ALT), and mean corpuscular volume (MCV).

Direct biomarkers of drinking have been developed more recently than indirect biomarkers. They are termed "direct" because they are analytes of alcohol metabolism formed by non-oxidative enzymatic processes (Karch, 2006). Although most alcohol consumed is metabolized by oxidative processes in the liver, a small amount is broken down non-oxidatively, creating analytes that can be measured for a longer period than when alcohol itself remains in the body. The direct markers that can be measured in the blood or urine include ethyl glucuronide (EtG) and ethyl sulfate (EtS), fatty acid ethyl esters (FAEE) and phosphatidylethanol (PEth). Characteristics of indirect and direct markers are summarized in Table 1.

There are two important factors, the sensitivity and the specificity, that must be considered when evaluating biomarkers. High sensitivity (ability to correctly identify all individuals who have consumed alcohol) and specificity (ability to correctly identify all individuals who have not consumed alcohol) are essential qualities of a good marker (Abudu,

2008). An ideal alcohol biomarker is 100% sensitive and 100% specific. However, the sensitivity and specificity can be influenced by other factors besides ethanol such as disease, smoking, gender, drugs or the diet. Sensitivity and specificity are calculated according to the formulas below:

$$\text{Sensitivity} = \frac{number\ of\ true\ positives}{number\ of\ true\ positives + number\ of\ false\ negatives}$$

$$\text{Specificity} = \frac{number\ of\ true\ negatives}{number\ of\ true\ negatives + number\ of\ false\ positives}$$

Table 1

Characteristics of Direct and Indirect Markers

Direct Markers	Indirect Markers
• Ethyl glucuronide (EtG) • Ethyl Sulfate (EtS) • Fatty acid ethyl esters (FAEE) • Acetaldehyde • Phosphatidylethanol (PEth)	• Aspartate aminotransferase (AST) • Alanine aminotransferase (ALT) • Gamma glutamyltransferase (GGT) • Mean corpuscular volume (MCV) • Carbohydrate-deficient transferring (CDT)
Elevated levels in light, moderate, and heavy drinkers	Elevated levels because of heavy alcohol consumption
Relatively high rates of elimination	Relatively slow elimination
Good indicators of recent alcohol consumption	Good indicators of extended periods of drinking
High specificity/sensitivity	Lack specificity/sensitivity

1.3 Phosphatidylethanol

An abnormal phospholipid in the brain, kidney, liver, and other organs was originally observed in 1983 in rats treated with ethanol (Alling, Gustavsson et al., 1983). This abnormal phospholipid was determined to be phosphatidylethanol (PEth) in 1984 (Alling, Gustavsson et al., 1984). Methods for quantifying PEth have become more advanced as a result of experiments conducted in both animals and humans (e.g., Alling et al., 1983, Rubin et al., 1988, Gustavsson

et al., 1991). These more reliable quantification methods have created opportunities to better understand the biomarker.

PEth is a phospholipid formed only in the presence of ethanol through the enzyme phospholipase D (Gustavsson, 1995). Like ethyl glucuronide, ethyl sulfate, and fatty acid ethyl esters, PEth is a direct alcohol biomarker. PEth is a molecule made up of a phosphoethanol head group and two fatty acid chains in the SN-1 and SN-2 positions of a glycerol structure. Each fatty acid typically contains 14 to 22 carbon atoms with 0 to 6 double bonds (Viel et al., 2012). There are many combinations of a fatty acid chain length and the number of double bonds in the fatty acids attached to the SN-1 and SN-2 positions of glycerol backbone. However, PEth16:0/18:1 (palmitic acid/oleic acid) and PEth16:0/18:2 (palmitic acid/linoleic acid) are two predominate homologues of 48 previously identified in human erythrocytes. PEth 16:0/18:1 and PEth 16:0/18:2 represent 37% and 25% of total PEth, respectively (Helander et al., 2009). In the current study, the pharmacokinetics of these two homologues will be further examined. The different PEth homologues are named after the fatty acid chains bound to the glycerol backbone (Gnaan et al., 2009).

Compared to the formation of PEth, the degradation rate is slower. PEth is detectable in the blood for up to 2-3 weeks with a half-life of about 4 days (Viel et al., 2012). The recognition of PEth and methods of detection, when considered within the context of other more established biomarkers, creates new opportunities to identify the quantity and different patterns of alcohol consumption. It has therefore become a promising new biomarker. The concentration of PEth seems highly correlated with the ethanol intake in alcohol users. Sensitivity and specificity of PEth are higher than those of other alcohol markers such as GGT and CDT (Isaksson et al., 2011). Several organizations are using PEth including numerous healthcare professional

programs, family courts, drug courts, Driving While Intoxicated/Operating While Intoxicated (DWI/OWI) court programs, and residential treatment centers (Dasgupta, 2015).

Interest in quantifying drinking patterns given an individual's PEth levels has become a focus of investigation. This information can be important for routine medical checkups and treatment for other related or unrelated medical concerns to alcohol consumption by identifying individuals consuming unhealthy amount of alcohol, during treatment for alcoholism to assess level of compliance, and in forensic settings to confirm legally imposed abstinence mandates (Dasgupta, 2015). The current study will examine the pharmacokinetics of PEth through in-vivo and in-vitro analysis of whole blood samples to understand whether one of the factors of PEth synthesis and elimination, the enzyme phospholipase-D, has an effect on PEth levels.

1.3.1 PEth Synthesis

Phosphatidylethanol is a unique phospholipid that is formed only in the presence of ethanol via the action of enzyme phospholipase D (Gustavsson, 1995). Normally, phospholipase D catalyzes the hydrolysis of phosphatidylcholine into phosphatidic acid. However, phospholipase D has a higher affinity for short-chain alcohols compared to water. Therefore, in the presence of ethanol, this enzyme promotes a transphosphatidylation producing phosphatidylethanol. In humans, two isoforms of phosphalipase D have been characterized. Phosphalipase D_1 is distributed perinuclearly and requires phosphokinase C activation while phospholipase D_2 is localized on cellular membranes. Both isoforms catalyze the formation of phosphatidylethanol on human red blood cell membranes. A study conducted by Aradottir, Moller, and Alling (2003) has shown that in-vitro incubation of whole blood with ethanol is directly proportional to the quantity of PEth produced. However, there were individual variations in PEth formation *in-vitro*. Arradottir and colleagues (2012) have stated that because inter-

individual differences in PEth formation are common, it may not be possible to correlate PEth in blood exactly to the absolute amount of ingested ethanol. This also holds true for other biological markers used as tests to monitor alcohol consumption.

1.3.2 PEth Degradation

The slower degradation of PEth compared to other biomarkers may be the result of the ineffiency of red blood cells. That is, the absence of phosphatidylcholine phospholipase C (PLC) activity in red blood cells may slow the degradation of PEth (Viel et al., 2012). PEth elimination has a half-life that varies between 0.5 to 2 hours in various cellular systems but has not yet been understood in red blood cells. In tissues other than whole blood the elimination rate of PEth is much faster (Lundqvist et al. 1994), which has may be caused by more active mechanisms of PEth degradation than are present in erythrocytes (Varga et al. 2000). Although phospholipases and PLC in-vitro release arachidonic and palmitic acid from PEth, there is still a lack of evidence of whether phospholipases are involved in in-vivo degradation of PEth (Viel et al., 2012) What has been understood is that there is a disproportion in PEth formation and degradation rates causing an accumulation of PEth within the cellular membranes allowing for PEth to be an indicator of chronic alcohol exposure (Viel et al., 2012). This disproportion in PEth formation and degradation could be caused by the enzyme activity of PLD which will be further examined in this study.

CHAPTER 2: HYPOTHESIS STATEMENT

2.1 Hypothesis

There will be a correlation between phospholipase-D activity in red blood cells and the rate of synthesis and elimination of phosphatidylethanol in-vitro.

CHAPTER 3: MATERIALS AND METHODS

3.1 Participants

We recruited two drinking groups: light (n = 27) and heavy (n =27) who were healthy participants between the ages of 21 and 40 with a BMI of ≤ 30. Participants had to report having had at least one alcohol drinking episode in the last month and an average intelligence (IQ ≥ 80) to ensure competence in understanding of the questionnaires and instructions. Exclusionary criteria for the study included a DSM-IV Axis I psychiatric disorder (including alcohol or other substance dependence); self-reported psychoactive medication use (last 3 months); pregnancy; a positive urine-drug test for metabolites of drugs of abuse (i.e. cocaine, amphetamines, methamphetamines, MDMA, opiates, or benzodiazepines) at study entry or during days where alcohol administration was scheduled to take place in the laboratory; respondents who showed evidence of alcohol withdrawal (CIWAAR scores > 10); pending criminal charges related to alcohol intoxication; a court mandate not to consume alcohol; a medical history that eliminates individuals for conditions that contraindicate alcohol consumption; and tampering with the alcohol monitor more than once during the study. Study volunteers were recruited using flyers, brochures, website advertisements such as Craigslist, and referrals.

3.1.1 Group Assignment

In this study heavy (> 3 drinks/day and > 7 drinks/week for women and > 4 drinks/day and > 14 drinks/week for men) and light (≤ 3 drinks/day and ≤ 7 drinks/week for women and ≤ 4 drinks/day and ≤ 14 drinks/week men) drinkers were recruited. Heavy drinkers must have had at least 2 days of abstinence per week, and light drinkers must have had only a single binge episode per week (defined as 4 drinks for women and 5 drinks for men consumed within 2 hours) (NIAAA, 2004). Light drinkers must also have reported having had at least one alcohol drinking episode in the past 28 days. Group classifications into heavy (0.8 g/kg) or light alcohol (0.4g/kg)

dose was assigned by chance. After enrollment, participants were required to remain alcohol abstinent for 7 days prior to the ethanol administration day in the laboratory which was ensured using a transdermal alcohol monitoring device.

3.2 Materials and Instruments

Structured Clinical Interview for DSM-IV Disorders (SCID). The SCID/NP is a semi-structured interview for DSM-IV Axis I diagnoses (First et al., 1996). This interview screened for Axis I psychiatric disorders and symptoms on a scale of 1 (absent), 2 (sub-threshold), 3 (threshold), or unclassified information). The SCID will be used to screen for Axis I psychiatric disorders.

Modified SCID for Substance Use (mSCID). The mSCID (Martin et al., 1995) is a modified version of the Structured Clinical Interview for DSM-IV Disorders used to assess lifetime patterns of substance use involvement. Data was collected on alcohol, marijuana, stimulant, sedatives, opioid, PCP, hallucinogen, and inhalant use. The mSCID assessed age of substance use onset and frequency of use to characterize lifetime patterns of drug use. Individuals regularly using drugs of abuse other than alcohol and marijuana or meeting criteria for substance dependence were excluded from the study.

Clinical Institute Withdrawal Assessment for Alcohol-Revised (CIWAAR). The CIWAAR is a ten item scale used in the assessment and management of alcohol withdrawal (Sullivan et al., 1989). Each item on the scale is scored independently, and the summation of the scores (range = 0-67) yields an aggregate score that correlates to the severity of alcohol withdrawal (Center, 2006). The ten items evaluated on the scale are common symptoms and signs of alcohol withdrawal, and are: nausea and vomiting, tremor, paroxysmal sweats, anxiety, agitation, tactile disturbances, auditory disturbances, visual disturbances, headache, and orientation. The

CIWAAR monitored symptoms of alcohol withdrawal and those individuals with scores ≥10 were excluded.

Time-Line Follow-Back Interview (TLFB). The TLFB was used to document self-reports of the type, quantity, frequency, and duration of alcohol use within a specified time period (Sobell and Sobell, 1992). This method is recommended for use when relatively precise estimates of drinking are necessary, especially when a complete picture of drinking days (i.e., high- and low-risk days) is needed (Dawes et. al, 2005 and Collins et. al, 2008). Although TLFB summary data are reliable, as with all self-reported drinking assessment methods, exact day-by-day precision cannot be assumed or expected. The TLFB characterized typical patterns of drinking for group assignment.

Health History and Medical Examination. A health history and medical exam was performed by the study's physician assistant who determined the eligibility of each volunteer's participation.

Expired-Air Alcohol Screening (Breathalyzer). The breathalyzer is a device used to measure the percent alcohol levels from an expired air breath sample by blowing into a disposable mouthpiece. This is an indirect and non-invasive way of estimating an individual's blood alcohol concentration (Vukovic et al., 2015). In this study Dräger Alcotest 6810 portable, hand-held breathalyzers (Dräger Safety Diagnostics Inc., Irving, TX) were used to measure breath alcohol concentrations.

Urine Drug and Pregnancy Screening. A urine sample was collected to test for drugs of abuse and/or pregnancy. The Panel/Dip Drugs of Abuse Testing Device, which screens for metabolites of cocaine, amphetamines, methampthetamines, MDMA, THC, opiates, and benzodiazephines, was used. For women, part of the urine sample was used to test for pregnancy with Redi

Screen™ Pregnancy Test Strips. Volunteers with either drugs in their urine (exception for THC) or a positive pregnancy test were excluded from participation.

Whole Blood Samples. Whole blood samples were collected at every visit using standard phlebotomy procedures. The whole blood was placed in EDTA Vacutainer™ tubes and mixed thoroughly to ensure that blood coagulation did not occur. The samples were then placed in a 4˚C refrigerator before being centrifuged and aliquoted into 3 polyprolene tubes for PEth analysis. The samples were then stored at -80˚C until assayed.

Transdermal Alcohol Monitor (SCRAM-II Device). The SCRAM-II (Secure Continuous Remote Alcohol Monitoring device) is a device worn on the ankle that measures transdermal alcohol concentrations (TAC) by using a fuel sensor to detect ethanol gas emitted through the skin. The device measures alcohol concentrations every 30 minutes until the readings are below 0.02% (Dasgupta, 2015). The device also records the temperature and skin reflectance using infrared light to monitor any tampering with the device. The data stored in the ankle bracelet was downloaded to a secure web server for analysis once per week. If tampering was detected, the participant received a warning; if it occurred again, he/she was discontinued from the study.

Alcohol Administration. Alcohol was administered by adding 0.4 or 0.8g/kg of 95% alcohol to 24 oz of fruit juice (orange, apple, mango, etc.). Participants were allowed 15 min consume the drink, which was divided into 3 equal parts (3 x 8oz) and administered at 5 min intervals to control for the rate of consumption.

High Performance Liquid Chromatography (HPLC). The whole blood samples were analyzed for PEth levels using HPLC and tandem mass spectrometry with minor changes made based on recently published methods (Zheng et al., 2011, Helander et al., 2012).

3.3 Procedures

3.3.1 Recruitment and Consent Procedures. In response to the community advertisements, participants contacted the researchers at the laboratory to inquire about the on-going study. Study personnel explained what the study was and how it worked via telephone. If participants were interested in the study, they gave verbal consent to follow through with the telephone screening. After the telephone screen, volunteers who appeared to have met the inclusion criteria were scheduled for their initial screening visit at the laboratory where they learned more about the study, and consented to participate if interested.

Upon arrival at the laboratory, participants read the informed consent packet which included the purpose of the study, information about the study procedures, requirements and length of participation, potential risks and benefits of participation, and compensation provided for participating in the study. Once the participant read the consent form, research personnel obtained informed consent from the participants. The volunteer was then provided a copy of the consent form and encouraged to ask questions at any point throughout the study.

3.3.2 Study Procedures. Once the participant signed the consent form, they were given several forms to fill out. These forms included tax exemption form, Four Factor Index of Social Status (Hollingshead, 1975), a medical history survey, and the Alcohol Use Questionnaire (Mehrabian and Russell, 1978). A breathalyzer test and a urine sample were required as well to determine recent alcohol use and any drugs in the system at that time. Once the forms were completed, research personnel completed a Time-Line Follow-Back interview to determine what the participant had to drink for the past 28 days, Structured Clinical Interview for DSM-IV Disorders (First et al., 1996), Modified SCID (Martin et al., 1995), and Clinical Institute Withdrawal Assessment for Alcohol- Revised (Sullivan et al., 1989). After completing the screening

questionnaires, the participant completed the health history and medical examination with the Physician Assistant on the same day or scheduled a time to come in for these assessments.

Participant qualification for study enrollment was determined from the screening instruments, the health history, and medical examination. If the participant met all the inclusion criteria, they were scheduled to return to the laboratory to be fitted with a SCRAM-II monitor that remained in place on their ankle for 22 days. They were also asked about their recent alcohol use since their previous visit.

The chronological design of the experiment is shown in Figure 1. On day 1 of the study participants were fitted to a SCRAM-II monitor. They were asked to return to the laboratory on Day 8 where they consumed a randomly assigned dose of alcohol. The visit began at 7:30am and ended at around 4:30pm depending on their BRAC levels. Participants were asked to not eat after midnight the night prior to returning to the laboratory. They were provided a meal around 2:30pm. On the day of their visit to the laboratory participants were asked to take a breathalyzer test to screen for recent alcohol use, and provide blood and urine samples. Data from the alcohol monitor was downloaded to SCRAM Net DirectConnect™, an online web server, to observe their alcohol patterns, if any, during the past week. They were also asked to report on their past week of drinking through the TLFB interview. Participants in the heavy and light drinking groups were randomized to consume either 0.4 or 0.8g/kg of alcohol. The alcohol was consumed within 15 minutes and blood was collected prior to and then again at 15, 30, 45, 60, 90, 120, and 360 minutes after the alcohol dose was provided. Participants had an IV catheter placed in their forearm to collect blood samples. Throughout the day blood was drawn from the IV placed in participant's arm. A small amount of blood was also taken from the finger at the time of the last arm blood draw. If for some reason and IV catheter was unable to placed in the participant's arm,

then blood was taken by individual sticks. After blood sampling was completed, the catheter was removed and participants remained in the clinic until sober.

On study days 9-22 there were 5 follow-up visits. They were asked to return to the laboratory on days 10, 12, 15, 19, and 22. During this 2 week time period they continued to wear the ankle monitor to verify abstinence outside of the laboratory. Each of the follow-up visits took less than an hour. During these visits data from the SCRAM-II monitor was downloaded and they were asked to provide breath and blood samples as well as report on their recent alcohol use.

On their last visit to the laboratory, after data from the SCRAM-II monitor had been downloaded and they provided breath and urine samples and reported on their recent alcohol use the ankle monitor was removed.

The blood and urine samples collected weekly measured PEth, carbohydrate deficient transferring (CDT), glutamyltransferase (GGT), urinary ethyl glucuronide (uEtG), and urinary ethyl sulfate (uEtS). Data gathered allowed for us to examine PEth concentrations to predict the pharmacokinetics of PEth synthesis and degradation.

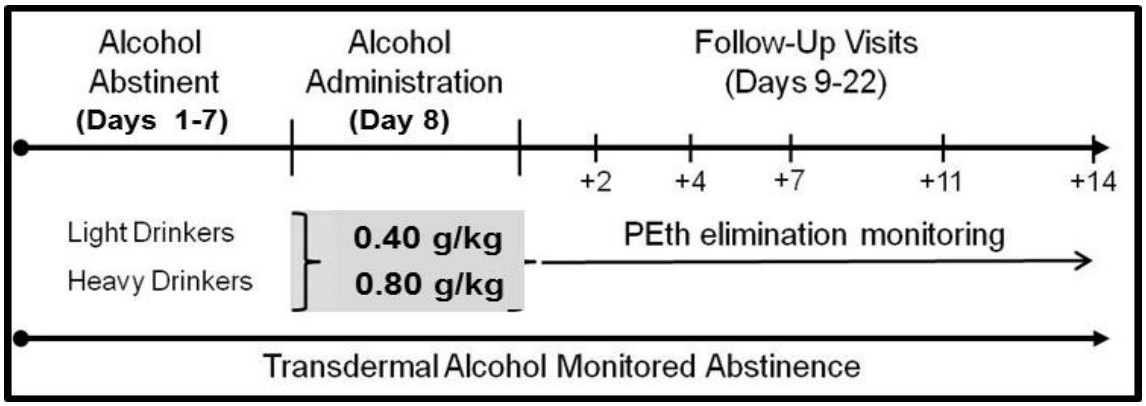


Figure. 1 Chronology of Study

3.4 Participant Compensation

All potential study participants were paid $10 in cash for completing the initial screening visit at the clinic. If the participant qualified for the study then additional compensation was provided. For the ankle monitor fitting day an additional $10 in cash was provided at the end of the visit. During the 22 days of the study, $10 per day was provided for wearing the transdermal alcohol monitor, $25 per day for each of the 5 follow-up visits to the laboratory, and a $300 bonus was paid for not drinking alcohol outside of the laboratory for the duration of the study. On the day the participant consumed alcohol in the laboratory, $5 was provided for arriving on time and $70 for IV blood draws. The total compensation at the end of the study was up to $740

3.5 Plan for Analysis

To test the hypothesis, a mixed model regression analysis and correlation between PEth vs. PLD activity was analyzed using GraphPad Prism software. In order to understand PEth vs. time, results were plotted in GraphPad Prism and understood through descriptive analysis. The hypothesis is that the PLD activity will have a correlation with PEth levels in-vitro suggesting that the enzyme is a factor in PEth synthesis and elimination.

3.6 Assay Procedures

Measurement of 16:0/18:1 and 16:0/18:2 in Whole Blood

PEth 16:0/18:1 and PEth 16:0/18:2, the two predominant homologues of PEth, were quantified in uncoagulated, whole blood samples using HPLC/MS/MS according to minor modifications of recently published methods (Helander et al., 2012; Zheng et al., 2011). The pharmacokinetics of both PEth homologues and the total of the two (combined PEth) were studied.

Measurement of PEth and Phospholipase-D Activity in Whole Blood

Phospholipase-D activity vs. PEth levels were quantified by performing an assay on 50 participants' baseline whole blood samples whose PEth AUC's varied over a relatively wide range. The samples must have had at least 500µl of whole blood sample to begin with in order to be analyzed. On the day of each assay, 10 participants' whole blood samples were thawed at room temperature. The samples took about 20-30 minutes to thaw. A 5M stock solution of ethanol was prepared by adding 30.7ml of 95% ethanol to 69.3ml saline. The stock solution was vortexed thoroughly and stored at room temperature. Then, seven 5mL test tubes that contained 325µL isopropanol, the solvent used to quench the reaction, for each of the 10 whole blood samples was prepared. At scheduled, staggered times 10µl of 5M ethanol stock solution was added to 490µl whole blood to obtain 500µl sample, and then the 500µl sample was vortexed for a few seconds to blend the ethanol solution with the whole blood. Immediately after vortexing the 500µl sample, 50µl was transferred to the 5mL test tube containing 325µL of isopropanol to obtain the baseline sample. The collected samples were placed in the refrigerator at 4˚C until all samples had been collected. Then, the remaining 450µl sample was placed in an incubator at 37˚C, removing 50µl of the sample every 60 minutes for 6 hours. Each of the 10 participant samples were staggered 2 minutes apart when removing 50µl from the 500µl sample to provide time for pipetting, vortexing, etc. Once all of the samples had been collected, the extraction procedure was completed by adding 500µl hexane to each sample. Then, the vials were gently shook for 20 minutes and centrifuged for 10 minutes at 1000g at 4˚C. The clear supernatant was transferred to new tubes and evaporated with nitrogen to residues at 30˚C. The residue was stored at -80^{o}C until the day of the assay for measurement of whole blood phosphatidylethanol.

On the day of the assay for PEth vs. PLD activity, 100µl of mobile phase A (20% 10mM ammonium acetate dissolved in water and 80% acetonitrile) was added to the residues which was then transferred to microfilterfuge tubes and centrifuged at 1000g for 10 minutes. The final samples were transferred to glass tubes and then 10ul was injected into the HPLC/MS/MS system where PEth was quantified by mass spectrometry according to minor modifications of recently published methods (Zheng et al., 2011; Helander et al., 2012). The remaining 40 participant samples followed the above procedures, with 10 participant samples assayed each day.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Results

Fifty-four participants were recruited into the study. Whole blood PEth levels were analyzed in all participants, however, in-vitro analysis of the relationship, if any, between whole blood PEth and phospholipase-D was analyzed in 50 participants. All the participants reported a regular pattern of drinking alcohol before they entered the study, and were able to provide negative TAC readings throughout the study indicating compliance with the abstinence requirement. After the first 7 days of negative TAC readings, blood samples were collected the morning of the alcohol consumption day. Detectable amounts of PEth were still seen in 53 of the 54 participants.

Combined PEth Levels on Ethanol Administration Day

After 0.4 and 0.8g/kg doses of ethanol were consumed, increases in combined PEth levels were observed (Figure 2) in every participant. Combined PEth levels reached a maximum around 90 to 120 minutes for both doses (Figure 2). Administration of single doses of 0.4 and 0.8g/kg of ethanol also showed PEth levels decrease post-alcohol consumption on the administration day after the 90 to 120 minute mark. There was a decrease in PEth levels as well as measureable amounts detected 2 weeks post-alcohol consumption, which was the last day of the study.

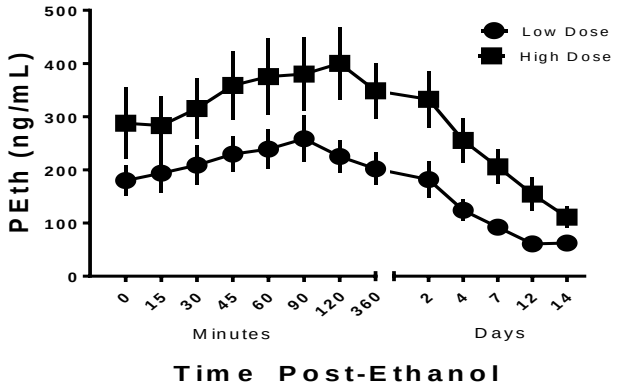


Figure 2. Concentration-Time Curves of PEth during Abstinence after Ethanol Consumption

Synthesis and Elimination of Individual PEth Homologues

The pharmacokinetic profiles of two PEth homologues, PEth 16:0/18:1 and PEth 16:0/18:2, and combined PEth during and after ethanol consumption are shown in figure 3. As indicated by the graph, PEth levels are at non-zero values since there were detectable amounts in many participants even after seven days of abstinence. The PEth levels were not adjusted to baseline levels. The mean concentrations of PEth 16:0/18:1, PEth 16:0/18:2, and combined PEth increased to a maximum at 90 to 120 minutes after alcohol administration and then decreased during the next 14 days of abstinence. As not only indicated by Figure 3, but also by Figure 4, which is representative example of the rate of PEth synthesis and elimination in one participant, PEth 16:0/18:1 and PEth 16:0/18:2 appear to have different rates of synthesis and elimination. PEth 16:0/18:2 is synthesized and eliminated at a faster rate compared to PEth 16:0/18:2.

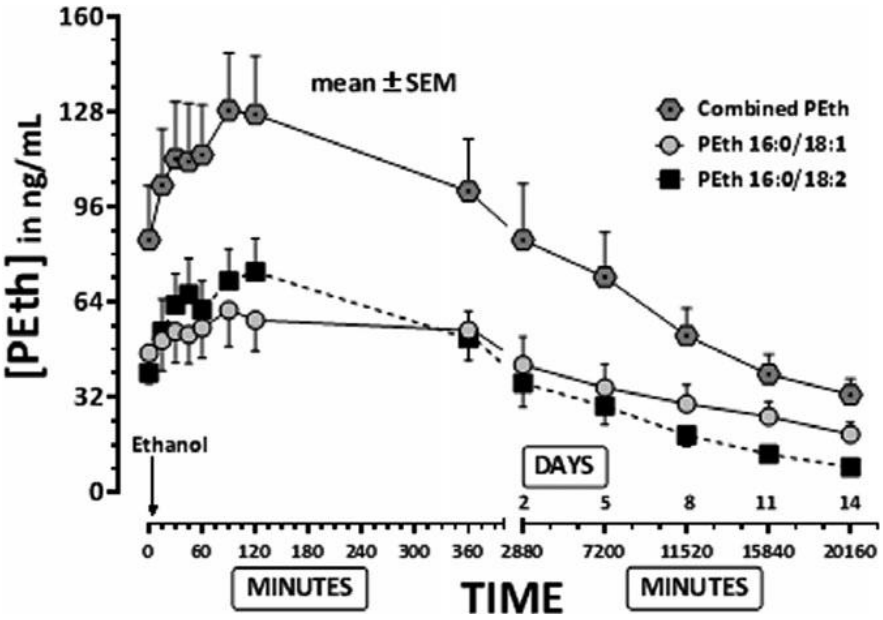


Figure 3. Concentration-Time Curves for PEth 16:0/18:1, PEth 16:0/18:2, and Combined PEth during Abstinence after Ethanol

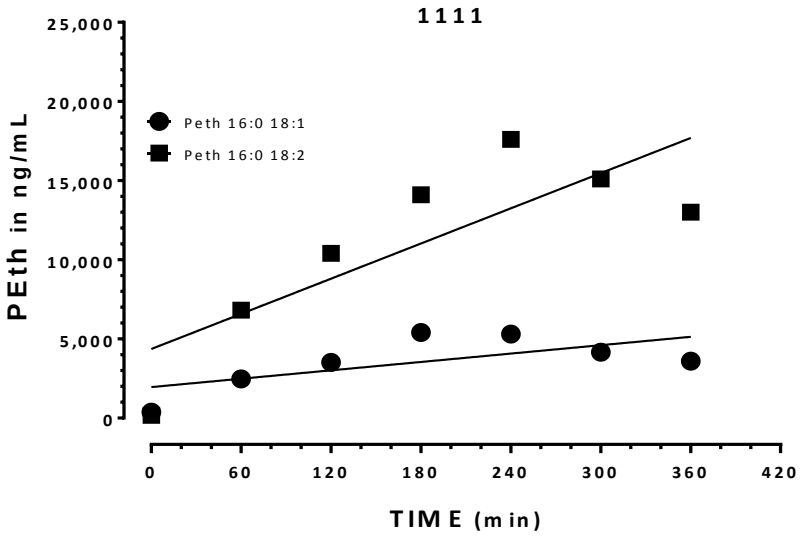


Figure 4. PEth 18:1-18:2 Rate of Synthesis and Elimination Over 360 Minutes

Correlation between Phospholipase-D and PEth

Baseline blood samples of 50 of the participants were used to understand the relationship between PLD activity and PEth AUCs through in-vitro analysis. Baseline PEth levels were non-

zero for 53 of the 54 participants. The r^2 value of 0.01 for PEth 16:0/18:1 and 0.002 for 16:0/18:2 indicates no level of significance between PLD enzyme activity and PEth AUCs (Figure 5).

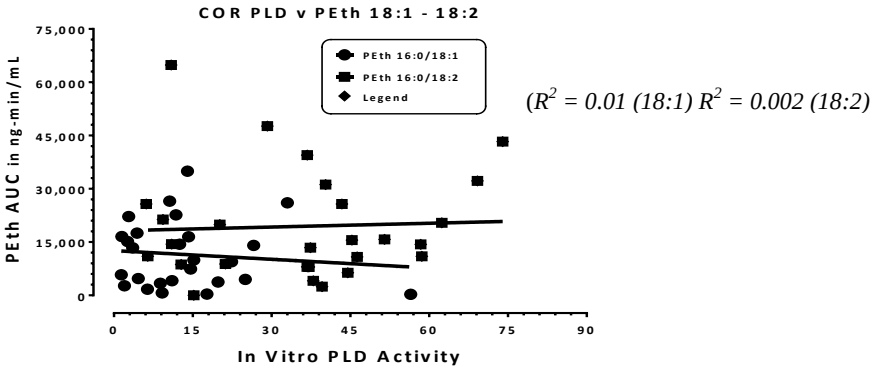


Figure 5. Correlation between In-Vitro PLD Activity and PEth AUC

4.2 Discussion

The results of this clinical laboratory study showed (i) administration of single doses of 0.4 and 0.8g/kg of ethanol showed PEth levels increased and decreased post-alcohol consumption on the administration day and was measureable 2 weeks post-alcohol consumption in every participant; (ii) the PEth homologue 16:0/18:2 is synthesized and eliminated at a faster rate compared to 16:0/18:1; (iii) there is no correlation between in-vitro phospholipase-D activity and PEth AUC.

In this study alcohol doses of 0.40 and 0.80g/kg were used to stimulate real world light and heavy drinking patterns. An increase in combined PEth levels after ethanol consumption was observed in every participant which indicates good sensitivity of this biomarker to detect low to heavy doses of alcohol in the blood.

Recent studies have suggested that different homologues of PEth are synthesized and eliminated at different rates during abstinence (Gnann et al., 2014; Helander and Zheng, 2009). The data collected from this study confirmed that the PEth homologue of 16:0/18:2 was synthesized and eliminated at a faster rate after ethanol consumption than the homologue

16:0/18:1. Also, it was observed that PEth 16:0/18:2 had a shorter half-life than PEth 16:0/18:1 during the two week period following ethanol consumption. Understanding the differential synthesis of the two homologues may be useful in estimating the last time an individual consumed a drink.

In order to further examine and explain the between subject variance and between dosage variance of PEth levels in participants, the PLD activity was measured in a subsection of the study cohort. It was noted in this study that PLD levels might actually not affect the rate of PEth synthesis (Figure 5) as there was no correlation seen between PEth AUC and the PLD enzyme activity. Additional factors that might account for PEth synthesis and elimination are noted in Figure 6. Some of the factors that might account for the differences in PEth synthesis are ethanol absorption in the body and the pharmacokinetics of phosphatidylcholine homologues (Viel et al., 2012). Ethanol absorption in the body can be affected by a multitude of factors including: female vs. male differences, age, body weight, liver size, genetic factors, such as variations in the enzymes that break down alcohol, and environmental factors, such as the amount of alcohol an individual consumes and his or her overall nutrition (NIAAA, 2004). Possible factors affecting the elimination rates of PEth include enzymes involved in elimination, half-life of RBCs, or half-life of PEth in platelets, lymphocytes, and RBCs. The enzyme affecting the elimination rates of PEth could possibly due to the absence of the enzyme phosphatidylcholine phospholipase C (PLC).The inability to efficiently degrade PEth in RBCs could be accounted for by the absence of this enzyme, however PEth degradation has still yet to be fully understood (Viel et al., 2012). PEth elimination rates can also be affected by the half-life of red blood cells because they are filtered and eliminated through the liver at the end of their lifespan. Therefore, the faster rate of PEth elimination might be explained by the liver breaking down red blood cells since there

would be less PEth accumulating on the membranes of red blood cells since that is where PEth is being measured. Lastly, the PEth half-life in platelets, lymphocytes, and RBCs might have an effect on the elimination rates due to the molecular variations as well as enzymatic differences in the 3 whole blood cell components (NIAAA, 2004).

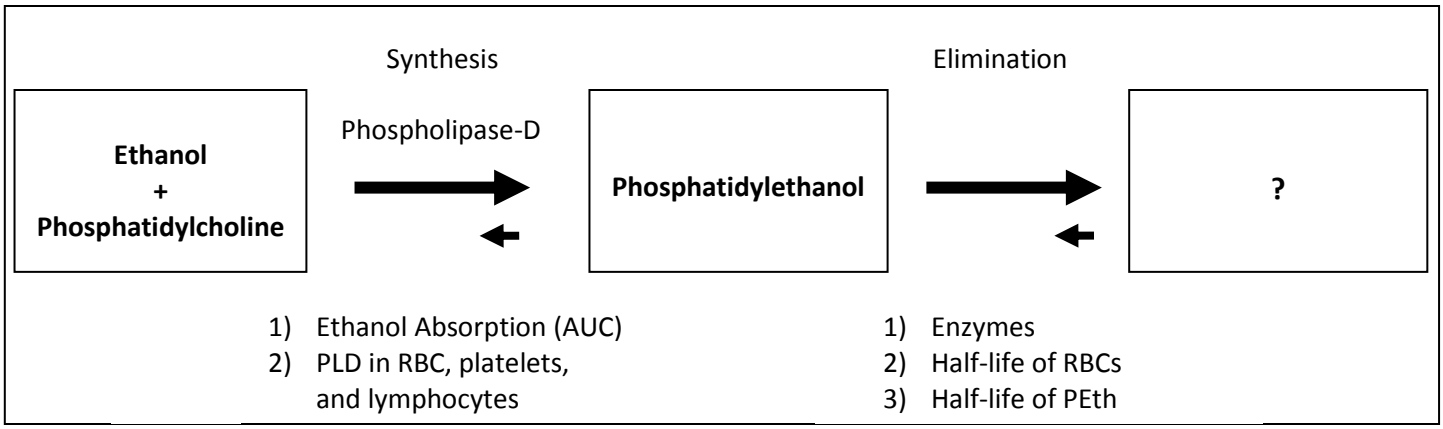


Figure 6. PEth Synthesis and Elimination Pathway
The diagram above shows the factors that can affect the rate of synthesis and elimination of PEth.

There were some limitations in the design of this study. First, sample size was limited and prevented in-depth comparisons of men vs. women, weight, and age categories. Second, many of the participants came in with detectable levels of PEth in their blood even after seven days of abstinence which could have affected precise calculations of PEth synthesis and elimination. Lastly, during in-vitro analysis of base-line whole blood samples saturating 490µl of blood with 10µl of ethanol might not represent real-world drinking patterns as a proportional concentration in in-vivo studies would be lethal, however a saturated amount of ethanol was used for study control purposes.

Future studies with a larger study sample and low to undetectable PEth levels at the start of the study would be preferred for a more comprehensive understanding of the pharmacokinetics of this biomarker. To continue to understand alcohol biomarkers such as PEth,

other studies should examine PEth AUC for the first 120 minutes, the time at which PEth levels reach maximum after ethanol consumption, and the PLD activity to see if there is a correlation of enzyme activity in the synthesis of PEth rather than examining both the synthesis and elimination as was done in this study. Studies should also be conducted on the other factors (Fig. 5) that affect PEth synthesis and elimination to see if there is a correlation between the factors and PEth AUC levels.

In summary, this study as well as recent studies on PEth continue to support the finding that PEth is a sensitive alcohol biomarker which when used along-side other alcohol biomarkers can hopefully predict individuals' recent alcohol consumption patterns. This will be clinically applicable in the future because it will provide a biological method to quantify potentially harmful ethanol consumption for medical and forensic purposes.

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Degree to which thesis applies: **BS** **N/A**

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1st Minor **Biology**

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2nd Minor

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