From the Department of Laboratory Medicine Division,
of Clinical Chemistry,
Karolinska Institutet, Stockholm, Sweden

DEVELOPMENT AND APPLICATION OF AN LC-MS METHOD FOR THE ALCOHOL BIOMARKER PHOSPHATIDYLETHANOL (PETH) IN BLOOD

Yufang Zheng

Karolinska Institutet

Stockholm 2011

Published by Karolinska Institutet.

ISBN 978-79-7457-599-6

“Den mätta dagen, den är aldrig störst.
Den bästa dagen är en dag av törst.”

Karin Boye

ABSTRACT

Objective biomarkers tracing alcohol consumption are demanded in many situations when alcohol drinking is in focus, e.g. during monitoring of patient in a treatment program, in forensic medicine, workplace testing or biochemical validation of self-report in research. Phosphatidylethanol (PEth) is an abnormal phospholipid formed only in the presence of ethanol that can be used as a sensitive and specific alcohol biomarker to detect current risky alcohol consumption. The aim of this project was to develop an liquid chromatography-mass spectrometry (LC-MS) method for PEth that is suitable for routine use.

PEth was extracted from whole blood and separated by LC-MS using a C4 column in a reversed phase system by gradient elution. The limit of detection ($S/N \geq 3$) and limit of quantification ($S/N \geq 10$) were ≤ 0.02 and ≤ 0.1 µmol/L, respectively. The calibration curve was linear in the concentration range 0.2-20 µmol/L and the intra-assay CV % for total PEth was ≤ 8.6 % and the inter- assay CV was < 11 %. The CV was lower using isotope labeled PEth as internal standard in the MS/MS mode.

Nine of the most common PEth forms were evaluated by both LC-MS and LC-MS/MS. PEth-16:0/18:1 and PEth-16:0/18:2 were found to be the major forms in blood from alcoholic patients. The correlations of PEth-16:0/18:1 and PEth-16:0/18:2 to total PEth were good ($R^2 = 0.973$) and PEth-16:0/18:2 ($R^2 = 0.983$) but together they correlated even better with total PEth. In 200 blood samples from blood donors and 3023 from the routine pool, the majority had a total PEth concentration ≤ 0.5 µmol/L. The amount of PEth formed in whole blood samples that were incubated in the presence of ethanol varied considerably between individuals. The value of PEth as an alcohol biomarker was compared with ethyl glucuronide (EtG), ethyl sulfate (EtS) and carbohydrate deficient transferrin (CDT) in an outpatient treatment program for alcohol-dependent subjects. Compared with CDT, PEth was found to be a more sensitive biomarker.

In conclusion, a sensitive and specific LC-MS method was developed for the routine measurement of PEth in whole blood samples. The measurement of PEth-16:0/18:1 alone or in combination with PEth-16:0/18:2 did not affect test sensitivity compared with total PEth. The use of PEth in combination with other biomarker is preferred, due to inter-individual variation in PEth formation.

LIST OF PUBLICATIONS

I. **Molecular species of the alcohol biomarker phosphatidylethanol in human blood measured by LC-MS.**
A. Helander, Y. Zheng.
Clin Chem 2009; 55: 1395-405

II. **Method development for routine liquid chromatography-mass spectrometry measurement of the alcohol biomarker phophatidylethanol (PEth) in blood.**
Y. Zheng, O. Beck, A. Helander.
Clin Chim Acta 2011; 412: 1428-1435

III. **Inter-individual variability of in-vitro formation of phosphatidylethanol (PEth) in blood.**
Y. Zheng, O. Beck, A. Helander.
(Manuscript)

IV. **Monitoring of the alcohol biomarkers PEth, CDT and EtG/EtS in an outpatient treatment setting.**
A. Helander, O. Péter, Y. Zheng.
(Submitted)

CONTENTS

LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CE-UV	Capillary electrophoresis with ultra violet detection
CDT	Carbohydrate-deficient transferrin
CV	Coefficient of variation
ELSD	Evaporative light scattering detector
EtG	Ethyl glucuronide
EtS	Ethyl sulfate
FAEE	Fatty acid ethyl esters
GGT	Gamma-glutamyl transferase
5-HTOL	5-Hydroxytryptophol
5-HIAA	5-Hydroxyindole-3-acetic acid
IS	Internal standard
LC	Liquid chromatography
LoD	Limit of detection
LoQ	Limit of quantification
MCV	Mean corpuscular volume
MS	Mass spectrometry
PA	Phosphatidic acid
PC	Phosphatidylcholine
PEth	Phosphatidylethanol
PProp	Phosphatidylpropanol
PLD	Phospholipase D
SIM	Single ion monitoring
SRM	Selected reaction monitoring
TLC	Thin layer chromatography

1 INTRODUCTION

Alcohol is a drug that is socially accepted and is deeply rooted in our society as far back as to the ancient history of mankind. People in many parts of the world are light to moderate drinkers and to them alcohol is not considered harmful. But chronic excessive alcohol intake leads to health and social problems to the individual [1]. To the society, the abuse of alcohol consumption not only increases the health care costs but also causes loss in productivity [2].

Chronic alcohol consumption leading to severe injury or death is a problem all over the world (www.can.se, www.who.int/mediacentre/factsheets/fs349/en/). Therefore, screening for alcohol related problems is an important task to detect early alcohol dependence or risky alcohol habits in connection to e.g. health care controls. This can be accomplished by structured interviews based on self-report [3, 4]. Due to the risk of underreport and denial leading to under-diagnosis of alcohol problems, laboratory tests offers a more objective method to trace alcohol intake and can be used as a complement to self-report measures [5, 6].

1.1 ETHANOL METABOLISM

The bioavailability is high for ethanol and it can access different organs quite readily. When absorbed in the gastrointestinal tract, ethanol is instantly distributed in the body. For men the volume of distribution is about 60 % and for women about 50 % body weight. The blood ethanol concentration after a given dose of alcohol varies from person to person depending on the body weight and water content. Usually women have less body water than men [7].

The major part (95 %) of the ingested ethanol is oxidatively metabolized in the liver following zero order kinetics. The elimination rate is constant with an average of about 0.1 g ethanol/kg body weight/hour. Hence, for a person with a body weight of 75 kg it takes approximately 10 h to metabolize the ethanol content in a bottle of wine (about 80 g). In the oxidation pathway ethanol is first degraded to acetaldehyde, catalyzed by the enzyme alcohol dehydrogenase (ADH). In the next step, acetaldehyde is further metabolized by aldehyde dehydrogenase (ALDH) to acetate. There is a genetic variation in ALDH causing deficiency in enzyme activity that is common in Asian populations. If these subjects consume alcohol it leads to accumulation of acetaldehyde that is very toxic to the body [8]. The oxidative metabolism also includes two minor pathways involving the enzymes CYP2E1 and catalase [9]. Besides, the

oxidative pathway, a minor part (1-2 %) of the ethanol is metabolized non-oxidatively producing metabolites containing the ethanol molecule, ethyl glucuronide (EtG), ethyl sulfate (EtS), fatty acid ethyl esters (FAEE) and phosphatidylethanol (PEth) (Fig. 1).

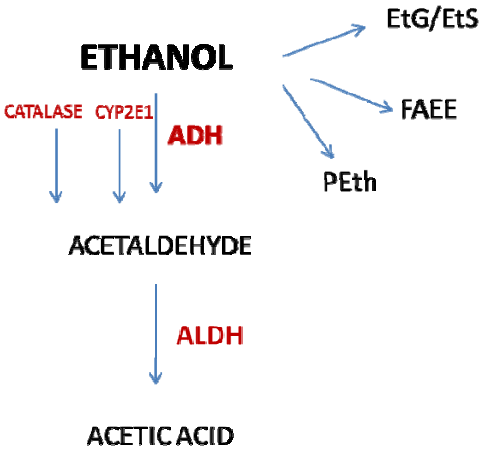


Fig.1. The major part of ethanol is metabolized by the oxidative pathway involving the enzymes ADH and ALDH. Only a small part is metabolized non-oxidatively producing the metabolites EtG, EtS, FAEE and PEth

Measuring ethanol in breath or in blood is a very specific method for detecting each ethanol intake [10]. A drawback is the short time window as ethanol is rapidly eliminated from the body [11]. Therefore, using alcohol biomarkers as indicators for ethanol intake offers a more extended time window ranging from days to weeks, depending on which biomarker is used and the amount of alcohol consumed.

1.2 ALCOHOL BIOMARKERS

Alcohol biomarkers can be divided in state and trait markers. Trait markers are inherited factors and predict future alcohol dependence [12] while state markers depend on prior alcohol consumption. Here, only state markers are considered which, are further divided in short-term and long-term alcohol markers. A short-term biomarker detects a single alcohol intake over the previous day/or days. Excessive alcohol consumption during at least a few weeks is usually a prerequisite for long-term alcohol markers to show a positive result.

There are two important factors, the sensitivity and the specificity that needs to be considered when evaluating biomarkers. An ideal alcohol biomarker is 100 % sensitive and 100 % specific. However, the sensitivity and specificity can be influenced by other factors than ethanol such as disease, smoking, gender, drugs or the diet. Sensitivity and specificity are calculated according to the formula below:

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

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

1.2.1 Short-term alcohol biomarkers

Ethyl glucuronide (EtG) and ethyl sulfate (EtS)

EtG and EtS are two conjugated ethanol metabolites that are stable and water soluble [13, 14]. Both EtG and EtS are useful as sensitive short-term alcohol markers that can be detected in urine even after a small ethanol intake. Depending on the amount consumed, EtG and EtS can be detected for up to 2-3 days after ethanol is not measurable [15-17]. Both EtG and EtS share the same excretion profiles and display the same sensitivity pattern [18]. As a biomarker for short-term alcohol intake, EtG and EtS are extensively used in routine clinical work [6,19,20].

5-Hydroxytryptophol (5-HTOL) and 5-hydroxyindole-3-acetic acid (5-HIAA)

During normal conditions most of the serotonin (5-HT) is metabolized to 5-HIAA and only a small part to 5-HTOL. However, under the influence of ethanol, these portions shift resulting in a higher concentration of 5-HTOL and this metabolic shift can be used as short-term alcohol biomarker [21]. As a biomarker it is better expressed the ratio of 5-HTOL/5-HIAA because this compensates for urine dilution and for the possible influence of dietary 5-HT [22]. 5-HTOL/5-HIAA is measured in urine [23], it correlates to ethanol intake [21] and has a maximum detection window of 24 h.

The only known substance that represses the 5-HTOL/5-HIAA ratio is Antabuse (disulfiram) an ALDH inhibitor used in alcohol detoxification therapy [24]. The 5-HTOL/5-HIAA test is not affected by age, gender and ethnicity or common diseases or medications [25]. Hence, the urinary 5-HTOL/5-HIAA ratio is a sensitive and specific

biomarker for short-term alcohol intake but the detection window is shorter compared with EtG and EtS [26].

Fatty acid ethyl esters (FAEE)

FAEE are produced from fatty acids and ethanol in an esterification reaction. FAEE are measured in blood and are detectable for at least 24 h after the last ethanol intake [27]. Also, FAEE can be measured in tissues in post-mortem sampling tracing ethanol intake prior to death [28]. As a short-term biomarker FAEE are rather sensitive and specific. There are gender differences where the FAEE level is more elevated in men [29].

1.2.2 Long-term alcohol biomarkers

Mean corpuscular volume (MCV)

MCV is the mean volume of the red blood cells and it has been extensively used in clinical practice as a long term biomarker for alcohol abuse. It takes months of heavy alcohol intake to increases the MCV value. After alcohol cessation it also takes long time to reach a normal MCV value, considering the life time for red blood cells is 120 days. The specificity of MCV is low for alcohol because other factors can also contribute to high values such as smoking, some medications or liver diseases [30].

γ-Glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

GGT, AST and ALT are liver enzymes measured in serum or plasma. The specificity and sensitivity of GGT, AST and ALT as alcohol biomarkers are low due to other factors such as hepatitis, liver metastasis, some medications or liver cancer that can also increase the enzyme levels. GGT, AST and ALT are poor biomarkers for early detection of risky alcohol consumption and are more used as indicators of liver dysfunction in general in a later phase [31, 32].

Carbohydrate-deficient transferrin (CDT)

Transferrin is a glycoprotein that is synthesized in the liver and transports Fe^{3+} ions in the body [33]. Normally, transferrin comprises of different glycoforms and following prolonged ethanol consumption, some glycoforms called carbohydrate-deficient transferrin (CDT) become elevated. One of these glycoforms, disialotransferrin is used diagnostically to evaluate alcohol abuse [34].

CDT is measured in serum and is extensively used in clinics as a long-term biomarker to indicate alcohol abuse in an early stage. The sensitivity of CDT as a biomarker of chronic alcohol abuse is high to moderate (Arndt 2001). However, the main advantage of CDT compared to liver enzymes is the very high specificity for long-term alcohol consumption. The half-life for CDT is 10-15 days and consuming 50-80 g ethanol daily in 1-2 weeks is considered required to become positive in CDT.

1.3 PHOSPHATIDYLETHANOL (PETH) AS AN ALCOHOL BIOMARKER

In 1983, during a study on phospholipids in rat organs after treatment with ethanol, an abnormal phospholipid was detected [35]. Later this abnormal phospholipid was characterized as PEth [36]. Subsequently, experiments were conducted both in animals and humans and PEth was quantified in various organs such as kidney, brain and the gastrointestinal tract [37, 38]. PEth was not detectable in blood from some animal species, [38]. However, PEth is present in both erythrocytes and leucocyte of human blood with the majority being membrane bound in red blood cells [39]. Attempts to detect PEth in plasma have not been successful [36].

As an alcohol biomarker, PEth has been detected in blood from heavy consumers for at least 14 days after cessation [40, 41]. Using a liquid chromatography-evaporative light scattering detector (LC-ELSD) method PEth is measurable in blood after consumption of 50 g ethanol/day for three weeks [42]. However, by a selective liquid chromatography-mass spectrometry (LC-MS) method, PEth is detected after one drink per day [43]. PEth and CDT correlate significantly with amount of ethanol intake where PEth is the most sensitive and specific metabolite [44].

PEth formation varies between individuals both for moderate and heavy drinkers [45]. Likewise, in-vitro study of PEth in whole blood that was incubated in the presence of ethanol indicted a substantial variation in PEth formation between samples [46, 47]. Hence, the PEth value cannot be used to determine the exact amount of previous ethanol intake, due to the inter-individual variability in PEth formation.

1.3.1 Formation and degradation of PEth

PEth is an ethanol metabolite and is formed from phosphatidylcholine (PC) only in the presence of ethanol by the action of the enzyme phospholipase D (PLD). PLD was first recognized in plants [48, 49] . The enzyme catalyzes the hydrolysis reaction of the membrane bound PC using water as substrate and producing phosphatidic acid (PA). A

new field opened for PLD when it was found in mammals and its involvement in a transphosphatidylation reaction having higher preference for ethanol as substrate over water leading to the formation of PEth at the expense of PA (Fig, 2) [50, 51]. PLD can be both membrane bound and free in the cytosol [52]. Both the membrane bound and the cytosolic form of PLD can catalyze PEth formation [53].

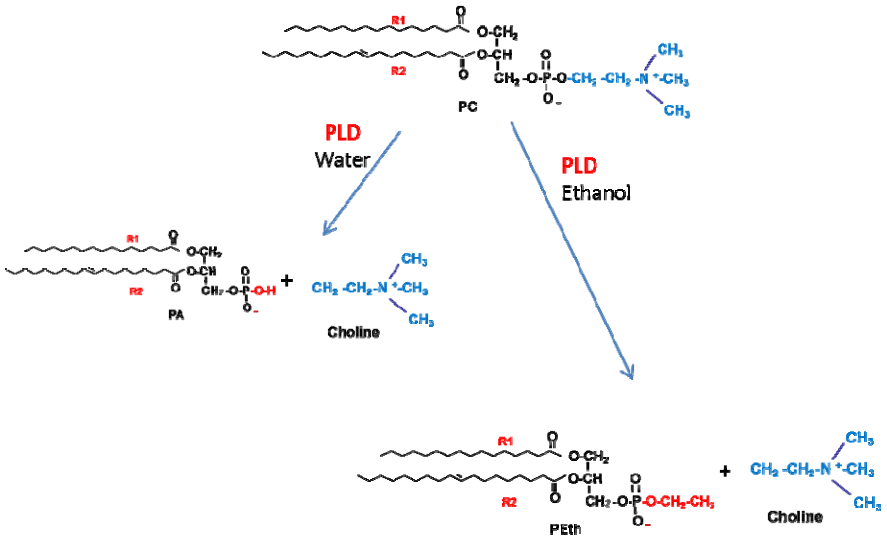


Fig. 2. Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) producing phosphatidic acid (PA) and choline, but in the presence of ethanol the transphosphatidylation reaction is preferred and phosphatidylethanol (PEth) is formed in the expense of PA. The R1 and R2 are fatty acid chains with varying length and degree of saturation.

In mammals two PLD isoforms, called PLD1 and PLD2, have been identified, with PLD1 having lower activity than PLD2 [54-56]. PLD is activated by signal molecules such as phosphatidylinositol 4,5-bisphosphate, monomeric GTP binding proteins, protein kinase C and Ca^{2+}, [57, 58]. PLD can be inhibited by molecules such as resveratrol and testosterone [57, 59].

Considering PC is the most common phospholipid in cell membranes (60-80 %), only 1-4 % is hydrolyzed to PA. PA is an intermediate molecule and is rapidly degraded to the signal molecules diacylglycerol and lysophosphatidic acid. In rat brain the PEth formation from PC was estimated to 0.05 % and in organs the degradation of PEth is faster compared to blood [60]. The half-life of PEth is reported to be 4 days and

the degradation path is not fully elucidated [39]. No gender difference has been identified for PEth elimination [61].

1.3.2 Molecular species of PEth

PEth is a lipophilic compound comprising of a glycerol head and two fatty acids of varying carbon length and saturation grade. Some of the common fatty acids are presented in Table 1. The structural composition of PEth is considered to mirror the molecular species of PC. There have been many studies on the structural composition of PC where chain lengths can vary from 14 to 24 carbons with the most common length (90 %) being 16 and 18 [62-64]. However, the molecular structure of PC is dependent on the fatty acid composition in the diet. A study on fatty acid intake from fish oil showed an increase in the fatty acids with a carbon chain of 20 and 22 by 3-4 mol % [62].

Table1.

There are many different ways of naming fatty acids with different chain lengths. Here a few of the more common fatty acids present in PC species are listed.

Common name	Chemical structure	C:D*
Palmitic acid	$CH_3(CH_2)_{12}COOH$	16:0
Oleic acid	$\mathbf{CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH}$	18:1
Linoleic acid	$CH_3(CH_2)_4\mathbf{CH{=}CH}CH_2\mathbf{CH{=}CH}(CH_2)_7COOH$	18:2
Arachidonic acid	$CH_3(CH_2)_4\mathbf{CH{=}CH}CH_2\mathbf{CH{=}CH}CH_2\mathbf{CH{=}CH}CH_2\mathbf{CH{=}CH}(CH_2)_3COOH$	20:4

*C = carbon numbers, D = number of double bounds

1.3.3 Bioanalysis of PEth

PEth was first detected using a thin layer chromatography (TLC) method. TLC was not suitable in clinical practice for PEth measurement and therefore, an LC-ELSD method was developed [40, 65]. The LC-ELSD method requires a long analysis time, a large sample volume, has relatively low sensitivity and measures all PEth forms ("total PEth") as a single chromatographic peak. An alternative PEth method using capillary electrophoresis with ultra violet detection (CE-UV) has been developed [66] and an antibody technique is under development [67, 68].

The measurement of PEth in whole blood is performed in a number of complex pre-analytical and analytical steps. The first step is to disrupt the cell membrane and

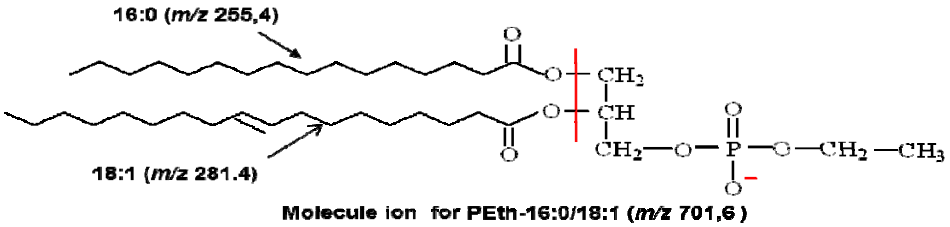
isolate lipids in an organic solvent. The organic phase will also contain other phospholipids present in whole blood. The chromatographic separation of PEth from other lipids is accomplished in the LC system.

Both LC-ELSD and CE-UV are non-selective methods that depend on the chromatographic separation and measures total PEth as a single peak. They are not able to separate contaminants that might co-elute with PEth. However, PEth molecules can be separately detected using selective single (MS) and tandem mass spectrometry (MS/MS) methods that measures molecules by their unique mass to charge value (*m/z*). In single MS, the single ion monitoring (SIM) mode is often applied that focus on the selected molecular ions for detection and quantification.

Molecules with identical molecular masses (e.g. for PEth-16:0/20:3 and PEth-18:1/18:2 (*m/z 725.6*)), can be separated by MS/MS which, mainly measures the unique product ions obtained from the deprotonated molecular ions. The most used method in MS/MS is the selected reaction monitoring mode (SRM). In SRM only the selected molecule (*m/z*) allows to be fragmented in a collision cell and then the selected product are measured. Fig. 3. shows the SRM mechanism for PEth-16:0/18:1.

MS/MS is a more sensitive and selective method than MS [69]. Thus, developing a simple LC-MS and MS/MS method for PEth measurement in routine increases the analytical sensitivity and improves the identification and the quantification standard.

a)



b)

Fig. 3. The general principle in SRM mode, a) the selected molecular ion is fragmented and b) the stable fragment ions (m/z), e.g. fatty acids 16:0 and 18:1 are measured.

1.4 METHOD VALIDATION

Method validation is a fundamental part in developing new analytical methods used either in the forensic-, or clinical laboratory or in research. The analytical method must be reliable and reproducible. The following validation parameters needed to be elucidated in a quantitative bioanalytical procedure according to the validation of ICH tripartite guideline (www.ich.org):

Calibration curve (linearity)

The calibration curve should be in the same matrix as the analytes and linear in the clinical relevant concentration range. At least six different concentrations should be included in the calibration. The standard deviation of the calibration curves based on six replicates needs to be evaluated.

Matrix effect

Different biological samples such as, serum, whole blood or urine in which the analytes are present can interfere in the ionization process by either enhance or suppress the dose-response signal. The matrix effect needs, therefore, to be considered in the method evaluation.

Reproducibility and repeatability

The variability of sample measurement between different days is expressed as reproducibility whereas repeatability is the imprecision of the same samples measured within the same day. The repeatability and reproducibility are investigated by repeated analysis of the same samples on different occasions. In this way various concentrations should be studied and at each occasion samples are prepared from scratch in triplicate prior to analysis.

Limit of detection (LoD) and limit of quantification (LoQ)

LoD is the lowest concentration detectable based on the analyte peak signal (S) that is three times higher than the base line, the noise (N; 3* S/N). LoQ is the lowest quantifiable concentration estimated as ten times the signal-to noise, (10* S/N).
LoQ can also be set at the lowest concentration with the coefficient of variation (CV) ≤ 20 %.

Recovery

Recovery is the percentage loss of the analyte during sample pretreatment prior to detection. If the analytes is in a complex matrix and requires sample clean-up by liquid-

liquid or solid phase extraction, the percentage loss of the analyte in the preparation steps needs to be considered.

Carry-over

Carry-over is a chromatographic problem caused by a previous sample with high concentration that is not totally washed away from the system and affects the next injected sample(s).

Stability

The stability is dependent on storage conditions and also how stable the analytes are during the sample preparation prior to analysis, such as multiple freeze-thawing cycles.

Impurities

Components from the assay that co-elutes in the chromatographic system together with the analyte should be evaluated. A pure substance is used to spike the impurity compound to indicate the retention time.

2 AIMS

- Development of PEth analysis using a selective LC-MS and LC-MS/MS method suitable for routine usage.
- To evaluate different PEth forms that are suitable to use in routine
- To compare stable isotope labeled PEth with phophatidylpropanol (PProp) as internal standards in the MS analysis.
- To study the inter-individual variation of PEth formation in vitro in whole blood in the presence of ethanol.
- To evaluate PEth as an alcohol biomarker in combination with other short-term and long-term biomarkers.

3 MATERIAL AND METHODS

3.1 COLLECTION OF WHOLE BLOOD SAMPLES

All blood samples used in paper I-IV were deidentified whole blood samples collected in EDTA tubes sent to the laboratory for PEth analysis, or of samples from blood donors. PEth was stable at least three weeks in blood samples if stored refrigerated. The procedure of using surplus volumes of anonymous blood samples for method development has been approved by the ethics committee at the Karolinska University Hospital.

3.2 EXTRACTION OF PETH FROM WHOLE BLOOD

To extract PEth from cell membranes, a solvent extraction method was applied. Whole blood (100 µL) was added drop wise to 600 µL isopropanol. The samples were then mixed using a shaker for 10 min. Subsequently, heptane was added (450 µL x 2) to extract the lipids to the organic phase. The samples were again extracted another 10 min on the shaker before centrifugation (10 min). The resulting organic phase was evaporated to dryness at 37 °C using nitrogen gas. The residue was dissolved in 50 µL heptane followed by 50 µL acetonitrile and 75 µL isopropanol. The aliquot was centrifuged (10 min) prior to LC-MS analysis. Noteworthy, since heptane was less toxic this solvent replaced hexane. The extraction procedure was based on previous publications [65, 70].

3.3 QUANTIFICATION OF UNKNOWN SAMPLES USING INTERNAL STANDARD TECHNIQUE

The MS quantification of PEth in samples was facilitated by the use of an internal standard (IS). The same amount of IS was added to the calibration, quality control and blood samples. Both the calibration and quality control samples were prepared in PEth negative whole blood. The dose-response signals from internal standard and samples were used to quantify PEth values. The concentrations used for quality controls were 0.1 and 6.85 µmol/L for PEth-16:018:1 and 16:0/18:2 standards.

3.4 SYNTHESIS OF ISOTOPE LABELED INTERNAL STANDARD (PAPER II)

PC-16:0/18:1 and PC-16:0/18:2 and deuterated ethanol were used to synthesize the corresponding PEth forms (PEth-16:0/18:1-d_5 and PEth-16:0/18:2-d_5) used as internal

standards. The reaction was started by adding the enzyme PLD. The reaction was stopped after 4 h by addition of acetonitrile that precipitates the protein.

3.5 INCUBATION OF WHOLE BLOOD (PAPER III)

In a first experiment, whole blood was incubated with ethanol in the range 0.25-2.0 g for 24 h, 48 h and 72 h, respectively. Then whole blood from clinical routine laboratory was incubated for 24 h and 1.0 g ethanol was added. All blood samples were incubated at 37 ºC on a heated metal block.

3.6 INSTRUMENTATION

LC-MS (paper I-IV)

The LC-MS system was an Agilent 1100 series. In MS the electrospray ionization (ESI) was used operating in negative ion mode. Analyte separation was achieved using a 50 x 3 mm, 5-µm HyPurity C4 column (Thermo Scientific) by gradient elution in a reversed phase system.

LC-MS/MS (paper I-II)

The LC-ESI-MS/MS system was a Perkin-Elmer series 200 LC system connected to Sciex API 2000 MS, with the ESI operated in negative ion mode and Analyst 1.1 software (Applied Biosystem).

Analysis of CDT (paper IV)

CDT was analyzed by an LC-UV method on an Agilent 1100 LC system. The transferrin glycoforms were detected at 470 nm [34].

Analysis of EtG and EtS (paper IV)

Both EtG and EtS were measured using an LC-ESI-MS method in SIM mode and negative ionization. The LC-MS system was an Agilent 1100 series [20].

4 RESULTS AND DISCUSSION

4.1 PAPER I

A method measuring PEth by LC-MS and LC-MS/MS was developed. The sample preparation was improved by decreasing the sample volume to100 µL whole blood and the solvent used was also minimized to a total of 1.5 mL that shortens the sample evaporation time. Using LC-MS the sensitivity was enhanced (LoD ≤ 0.02 µmol/L and LoQ ≤ 0.1 µmol/L) compared to the LC-ELSD method. The method validation was accomplished according to the ICH tripartite guideline**.** The calibration curve was linear in the concentration range 0.2-20 µmol/L and the intra-assay CV% for total PEth is ≤ 8.6 % and the inter-day assay was < 11%. There was no carry-over detected in LC-MS and the matrix effect was also absent. PEth was stable on storage at -80 ºC for at least 14 months. The total PEth amount produced by LC-MS method was compared to the LC-ELSD method used in routine for PEth analysis. Both methods displayed similar results in the lower concentration range (< 3 µmol/L) whereas the LC-MS method generally gave higher values above this threshold.

Using blood samples from alcoholic patients, a total of nine different PEth molecular species were identified and by LC-MS (Fig. 3).

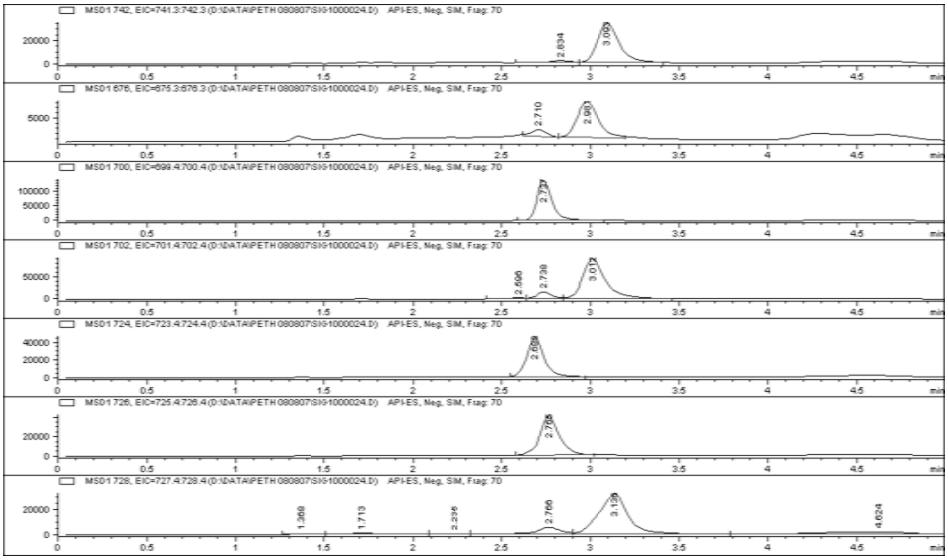


Fig. 4. An LC-MS chromatogram of an extracted PEth positive whole blood sample. In SIM, PEth molecules with identical masses are not separated but measured as a single peak. For example, PEth-16:0/20:3 and PEth-18:1/18:2 share a common molecular mass of m/z 725.6.

All PEth forms were further evaluated by MS/MS in SRM mode that measures the fragmented fatty acids. Some PEth forms that have identical masses were separated by their unique fragment ions see (Table 2). PEth was detected in all samples (n = 39) from heavy alcohol consumers and PEth-16:0/18:1 and PEth-16:0/18:2 were the most dominated forms.

Table 2.

In SIM the selected PEth molecular ions are measured by their individually masses and in SRM mode the stable fragment ions are measured.

PEth molecular species	SIM (*m/z*)	SRM (*m/z*)
PEth-16:0/16:0	675.6	255.6 (16:0)
PEth-16:0/18:2	699.6	255.6 (16:0) 279.5 (18:2)
PEth-16:0/18:1	701.6	255.6 (16:0) 281.6 (18:1)
PEth-16:0/20:4	723.6	255.6 (16:0) 303.5 (20:4)
PEth-16:0/20:3, PEth-18:1/18:2	725.6	255.5 (16:0) 305.5 (20:3) 279.5 (18:2) 281.5 (18:1)
PEth-18:1/18:1	727.6	281.5 (18:1)

The PEth forms measured by LC-MS and MS/MS in this study were selected from the composition of PC molecular species in human erythrocytes. In agreement with previous studies on the species distribution for PC, the present study concluded the PEth-16:0/18:1 and PEth-16:0/18:2 were the predominate forms in human whole blood. Thus, the PEth formation from PC is apparently not limited to certain molecular species.

For clinical use of LC-MS and MS/MS methods measuring PEth, focusing only on PEth-16:0/18:1, alone or in combination with PEth-16:0/18:2, is recommended. Also, the cut-off value of 0.7 μmol/L for total PEth that is presently used in clinical practice by LC-ELSD method might need to be reconsidered, since the MS methods are able to measure much lower PEth levels (LoQ ≤ 0.1 μmol/L).

4.2 PAPER II

In this assignment the focus was on PEth-16:0/18:1 and 16:0/18:2. The method validation was accomplished in SRM mode in MS/MS according to the ICH tripartite guideline**.** The LoD was 0.009 µmol/L and LoQ was 0.03 µmol/L for the fragment ions *m/z* 281 and *m/z* 279 for PEth-16:0/18:1 and 16:0/18:2 respectively. In the matrix study, the post-column flow test revealed no matrix contribution. However, when comparing the signals with and without matrix, there was a significant signal enhancement on average 43 % (n = 10). This matrix effect was corrected by the IS and the quantified PEth value is the same in the buffer compared to in the prepared blood samples.

The analytical imprecision was much lower when using deuterated PEth compared to phosphatidylpropanol (PProp) as IS in SRM mode. Independent calibration curves based on the PEth-16:0/18:1 and PEth-16:0/18:2 were prepared using the isotope labeled analogs as IS. The PEth values obtained in SRM and SIM were in good agreement (6 replicates covering the concentration range 0-7 µmol/L), for PEth-16:0/18:1 ($y_{SIM} = 1.056x_{SRM}-0.083$, $R^2 = 0.973$) and PEth-16:0/18:2 ($y_{SIM} = 1.004x_{SRM}+0.001$, $R^2= 0.983$).

The correlation of PEth-16:0/18:1 to total PEth was good but an even better correlation was seen in the combination of PEth-16:0/18:2 and 16:0/18:1. The most sensitive PEth form was PEth16:0/18:1 (100 % sensitivity) (Fig. 5).

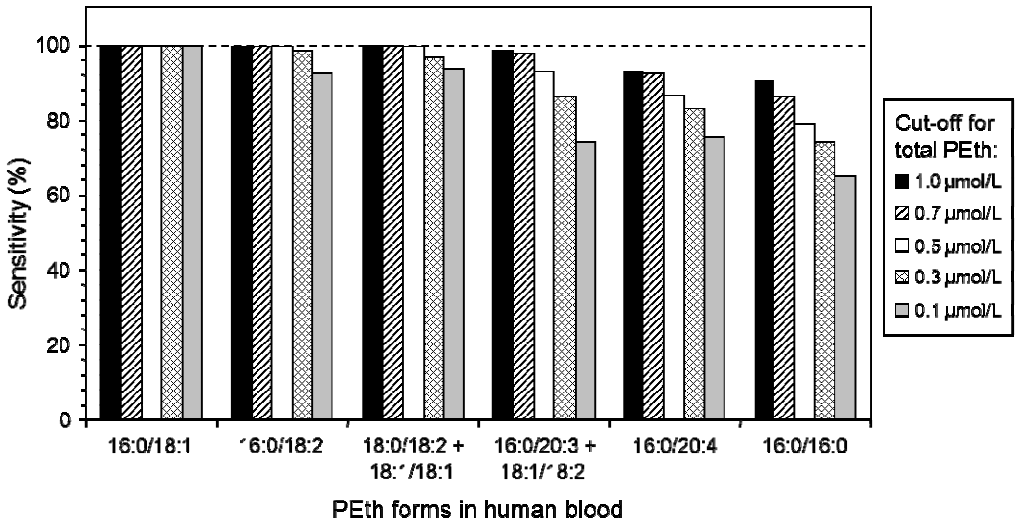


Fig. 5. The sensitivity of individual PEth forms at different cut-offs were evaluated for 211 blood samples measured by LC-MS. PEth-16:0/18:1 was the overall most sensitive form.

Blood samples from blood donors (n = 200) (Fig. 6) typically displayed low PEth values ≤ 0.5 µmol/L. However, in samples from the routine sample pool (n = 3023), much higher concentrations were detected.

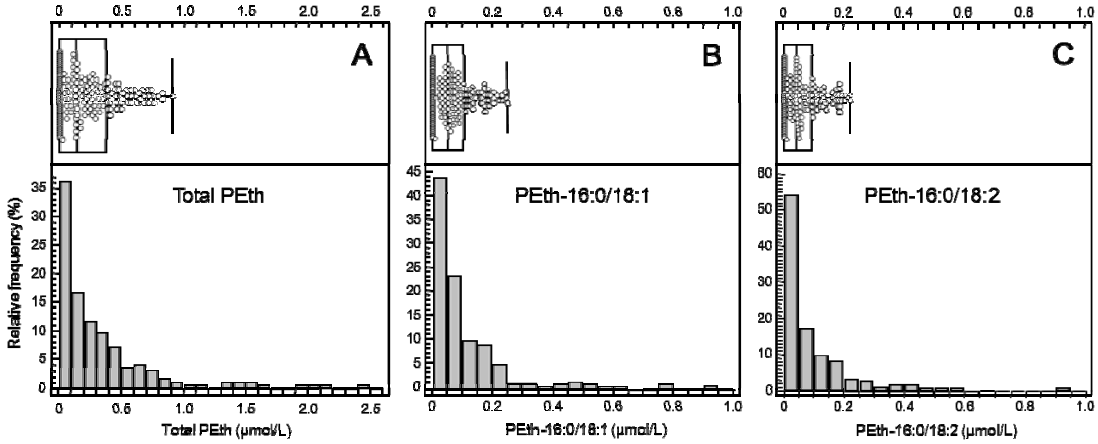


Fig. 6a. The distribution of A) total PEth and individual PEth forms B) PEth-16:0/18:1 and C) PEth-16:018:2 in samples from blood donors.

The use of isotope labeled analogs for PEth-16:0/18:1 and PEth-16:0/18:2 as internal standards were recommended in MS/MS, due to the variation in fragment ion signals in SRM mode. Also, this study demonstrated good correlations for PEth-16:0/18:1 and 16:0/18:2 independently to total PEth. However, the sum of PEth-16:0/18:1 and 16:0/18:2 were even better and were recommended as the target PEth forms in clinical practice. This test strategy might also compensate for the obvious inter -individual variation in the PEth profile. Additional study should be carried out on the diagnostic sensitivity and specificity to settle whether measurement of one or two PEth forms is enough or if the total amount is preferable for clinical usage. However, estimating total PEth from one species (e.g. PEth-16:0/18:1) by using a conversion factor will obviously not affect test accuracy.

The results from the blood donor samples and the routine samples, were used to suggest a reference intervals for PEth-16:0/18:1 and PEth-16:0/18:2. Because MS methods are more sensitive than the previously employed LC-ELSD method, it enables detection of even low-moderate drinking. Hence, a new reporting limit should be applied to indicate different alcohol consumption levels.

4.3 PAPER III

In a first set of experiments, the PEth formation was found to correlate linearly to the ethanol concentration (0.25-2 g/L ethanol) following incubation at 37 ºC. At an ethanol concentration of 0.5 g/L and higher, the PEth formation increased linearly with the increasing ethanol amount up to 24 h (Fig. 8). At higher ethanol concentration and longer incubation times, the PEth formation was indicated to level off.

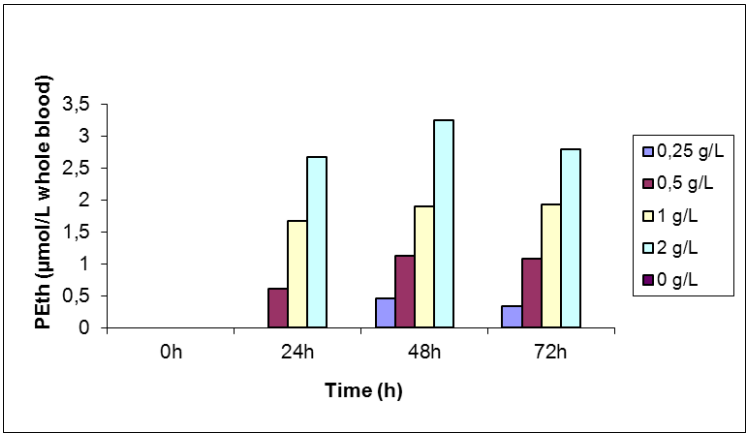


Fig. 7. The PEth formation increased linearly with increasing ethanol concentration. It seems that. Incubation time longer than 24 h had little effect on PEth formation. At each time interval, a zero control of a blood sample was incubated.

Hence, the inter-individual variability of the PEth formation in whole blood was studied in 46 blood samples that were incubated at 37 ºC for 24 h in the presence of 1 g/L ethanol. The initial PEth concentration in the samples (0-3.0 µmol/L) was corrected to get the actual PEth amount formed. The amount PEth formed was in the range 0-0.50 µmol/L with a mean value of 0.21 µmol/L and median of 0.23 µmol/L.

The in-vitro formation of PEth varied considerably between different samples, according to previous work. However, in this experiment a clinically more relevant ethanol concentration was used and a much larger number of blood sample were investigated. An interesting observation in this study was that some samples seemed not to form PEth or only trace amounts after incubation in the presence of ethanol.

Whether this is really the case or an artifact due to the design of the in-vitro experiment needs to be followed up in further studies. Clinical studies involving PEth have indicated a very high sensitivity (at or close to 100 %) of this alcohol biomarker [45, 61, 71]. The formation of PEth in human blood is lower (<0.01 %) than in rat brain (0.05–0.1 %) [60]. This could depend on lower PLD enzyme activity in blood

compared with in organs. High PEth concentrations are detected in various organs in human post-mortem samples [37].

Table 3.

CDT and PEth values on admission and during outpatient treatment

Biomarkers	**On admission[1] n (%)**	**During treatment[2] n (%)**
Cutoffs: CDT (>1.7 %) and total PEth (>0.1 µmol/L)[3]		
CDT and total PEth negative	11 (27.5 %)	9 (34.6 %)
CDT and total PEth positive	13 (32.5 %)	9[4] (34.6 %)
CDT positive, PEth negative	1 (2.5 %)	
CDT negative, PEth positive	15 (37.5 %)	8 (30.8 %)
Cutoffs: CDT (>1.7 %) and total PEth (>0.7 µmol/L)[3]		
CDT and total PEth negative	17 (42.5 %)	13 (50.0 %)
CDT and total PEth positive	13 (32.5 %)	8[4] (30.8 %)
CDT positive, PEth negative	1 (2.5 %)	1[5] (3.8 %)
CDT negative, PEth positive	9 (22.5 %)	4 (15.4 %)
Cutoffs: CDT (>1.7 %) and PEth-16:0/18:1 (>0.2 µmol/L)[3]		
CDT and PEth-16:0/18:1 negative	13 (32.5 %)	12 (46.2)
CDT and PEth-16:0/18:1 positive	13 (32.5 %)	8[4] (30.8 %)
CDT positive, PEth-16:0/18:1 negative	1 (2.5 %)	1[5] (3.8 %)
CDT negative, PEth-16:0/18:1 positive	13 (32.5 %)	5 (19.2 %)

[1] All patients (N = 40).

[2] Only patients from which ≥3 blood samples were obtained (N = 26).

[3] The cut-offs used to indicate a positive test result was >1.70 % for CDT (% disialotransferrin) in serum [72, 73], and >0.10 µmol/L (any drinking) and >0.70 (excessive drinking) for total PEth and >0.20 (excessive drinking) for PEth-16:0/18:1 in whole blood [74].

[4] One of these patients consistently showed incomplete separation between disialo- and trisialotransferrin (i.e., a C2C3 genotype or di/tri-bridging pattern) [72, 75] that prevented reliable quantification of the % disialotransferrin level.

[5] In this female patient, pregnancy was the likely cause for two borderline positive CDT samples during the third trimester [76]. Immediately after the delivery, her values returned to normal.

4.4 PAPER IV

Urine and blood samples for measurement of the alcohol biomarkers EtG/EtS, CDT and PEth were collected over two years from 40 outpatients with alcohol problems. Table 3 summarizes the CDT and PEth values on admission and during treatment.

The initial value for total PEth ranged between 0-16.5 µmol/L and in the end of the treatment the amounts had decreased to 0-5.9 µmol/L (14 of the 26 cases leaving at least 3 blood samples were included). For PEth-16:0/18:1, the starting values were 0-4.7 µmol/L and at the end 0-2.3 µmol/L. For CDT, the relative (%) disialotransferrin-to- total transferrin value in the beginning ranged between 0.87-6.9 % and at the end were reduced to 0.87-3.3 %. The individual results for total PEth, PEth-16:0/18:1 and CDT measured initially and at the end of the treatment program are illustrated in Fig. 8.

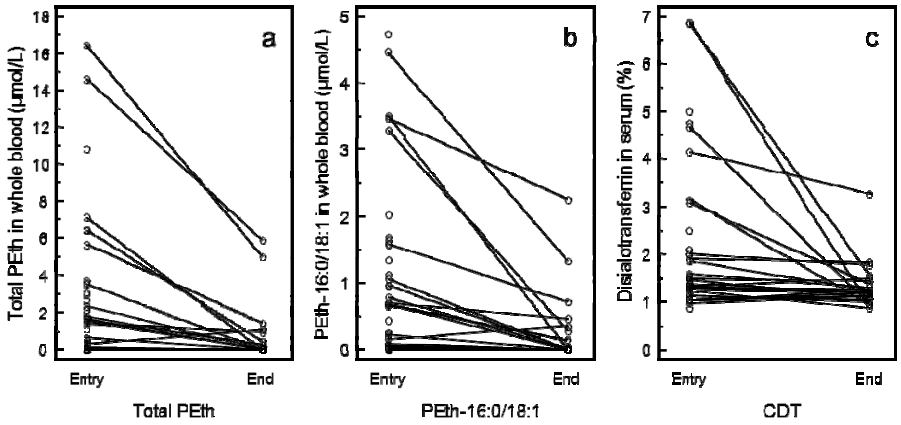


Fig.8. Individual changes in (a) whole blood total PEth, (b) PEth-16:0/18:1 and (c) serum CDT (% disialotransferrin) at start and in the final samples. The results for 26 patients who gave at least 3 blood samples are connected with lines.

The short-term biomarkers EtG/EtS were positive on several occasions during outpatient treatment. Since both EtG and EtS are direct ethanol metabolites, a positive result is a reliable indicator of a single small-moderate alcohol intake. Interestingly, the relapse rate was even lower among the patients who were also leaving urine samples for EtG and EtS, in addition to blood for PEth and CDT. In this study, PEth was able to detect more positive samples than CDT, due to the fact that the very sensitive LC-MS method is able to detect low-to moderate drinkers.

However, the substantial inter-individual variation in PEth formation complicates the diagnosis of risky drinking using this test alone. The combination of PEth with short-term and long-term biomarkers appears more valuable. Measuring PEth-16:0/18:1 alone instead of total PEth did not affect the test sensitivity significantly. To be able to focus only on one PEth subform facilitates the LC-MS method and opens for future standardization of the PEth assay.

5 GENERAL DISCUSSION

PEth is a metabolite formed only in the presence of ethanol. Previously, the measurement of PEth in whole blood in clinical practice was accomplished using an LC-ELSD method that was developed in 1998. Both the pre-analytical and analytical steps were complicated and required a large sample volume and long analysis time. The quantification of PEth was also complex. LC-MS method for quantification of PEth was only used in research and for evaluation purposes.

In our first paper, in 2009 we developed an LC-MS method for PEth measurement in clinical routine use. By LC-MS the different PEth forms are separated and selectively detected, and 48 different PEth homologues have been identified in autopsy material from a heavy drinker [77]. When measuring PEth in routine, the two common forms PEth-16:0/18:1 and PEth-16:0/18:2 were most prominent. Nevertheless, focusing on only one or two out of all PEth forms in routine by LC-MS could be problematic when encountering situations where PEth-16:0/18:1 or PEth-16:0/18:2 were not the dominating forms.

The fatty acid composition of PC, which is the precursor of PEth, is influenced by the fatty acid intake from the diet. No scientific report has yet been conducted on the indirect effect of the PEth forms by nutritional intake. For example, vegans and vegetarians are two groups that get most of the fatty acids from fish oil and vegetables with other fatty acids chain lengths than 16 and 18 carbons. Thus, ethanol consumption by people on special diets needs to be investigated for the common PEth forms by LC-MS [78].

The interest of using PEth in blood as an alcohol biomarker is growing and new methods have been developed on more selective instrument [79-82]. The LC-MS method increases the sensitivity of PEth analysis leading to a detection of low concentrations and is thereby able to pick up moderate alcohol consumers. A new reporting limit needs to be determined that distinguish low-moderate to heavy alcohol consumers. Another aspect that also should be investigated is compounds that inhibit PLD, since it has a direct effect of the PEth formation. Also, the present LC-MS method needs to be further improved for routine use by decreasing the total analysis time and facilitate the extraction procedure for PEth from whole blood.

6 CONCLUSIONS

- A simplified LC-MS and LC-MS/MS method suitable for routine PEth analysis was developed. Both sample pre-treatment and the analysis time were facilitated using MS detection. By MS, PEth-16:0/18:1 and PEth-16:0/18:2 were mainly evaluated.
- PEth-16:0/18:1 and PEth-16:0/18:2 alone displayed good correlation to total PEth but together the correlation was even better. However, in clinical samples both from blood donors and the routine sample pool, PEth-16:0/18:1 was the most sensitive form for alcohol consumption.
- The test sensitivity was increased by LC-MS allowing for detection of much lower PEth concentrations.
- The % CV was much lower using isotope labeled PEth compared to PProp as internal standard in SRM mode.
- Incubation of whole blood at 37 °C in the presence of ethanol displayed significant variation in the PEth formation between different samples.
- PEth was indicated to be a more sensitive alcohol biomarker than CDT. However, in routine clinical use, PEth may preferably be combined with other long-term (CDT) and/or short-term (EtG/EtS) biomarkers.

7 ACKNOWLEDGEMENTS

Life is the past, present and the future, so here it goes:

Anders Helander, my main supervisor, for founding this project and inviting me to the world of alcohol markers. Thank you for all the help and support. I'm glad that you talked me in to doing a PhD.

Olof Beck, my co-supervisor, you are the busiest man I known; still you are taking time to give me constructive criticism and valuable opinion both in the practical work and in writing this thesis. Thanks! I'm glad I met you in this life; otherwise I would miss out knowing a man that uses computer and type with only one finger.

Ann-Charlotte Bergman, my mentor, for helping me in method development in the first part. Thank you for taking the time and sharing your knowledge with me.

Present colleagues at Alcohol laboratory

Naama Kenan, Dara Degan and Josefine Lemon, keep the lab floating and Good Luck to you all!

Present colleagues at clinical chemistry

I'm glad that I've met so many nice people in Huddinge and have been part of a group that is both warm and open.

Veronika Tillander (I'm going to miss gym sessions with you, good luck and keep in touch!), *Xiao-Li Hu* (you really set a high level in presentation technique. You rock!), *Tina Kannisto* (very focused and ambitious), *Jenny Bernström* (for always being helpful with a big smile, your lamb are the best). *Sissel Kulstadvik , Matteo Pedrelli , Lilian Larsson, Maria Olin, Anita Lövgren Sandblom, Ulla Andersson, Maura Heverin, Gösta Eggertsen, Paolo Parini, Stefan Alexson, Ingemar Björkhem* and all others, for being nice colleagues and always helpful.

Former colleagues at alcohol laboratory

Jonas Bergström and Christian Björnstad. The good old days were when you guys were still part of the alcohol lab.

Helen Dahl, it is an experience to move with you from Solna to Huddinge. I've never met someone so organised and handy.

Former coffee friends at surfactant laboratory

Bim Linderholm and Marie Haegerstrand-Björkman, for endless discussions and all the laughs during the coffee breaks.

Former colleagues at CMMS

Rolf Wibom, for excellent guidance at my first job as an analytical chemist at CMMS. Thanks!

Vahid Edrisi, for being a nice colleague and making my time at CMMS more fun.

8 REFERENCES

1. Rehm, J., et al., *Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders.* Lancet, 2009. **373**(9682): p. 2223-33.
2. Jarl, J., et al., *The Danish effect on Swedish alcohol costs. An analysis based on hospitalization data from southern Sweden.* Eur J Health Econ, 2006. **7**(1): p. 46-54.
3. Allen, J.P., et al., *A review of research on the Alcohol Use Disorders Identification Test (AUDIT).* Alcohol Clin Exp Res, 1997. **21**(4): p. 613-9.
4. Mayfield, D., G. McLeod, and P. Hall, *The CAGE questionnaire: validation of a new alcoholism screening instrument.* Am J Psychiatry, 1974. **131**(10): p. 1121-3.
5. Feunekes, G.I., et al., *Alcohol intake assessment: the sober facts.* Am J Epidemiol, 1999. **150**(1): p. 105-12.
6. Dahl, H., et al., *Urinary ethyl glucuronide and ethyl sulfate testing for detection of recent drinking in an outpatient treatment program for alcohol and drug dependence.* Alcohol Alcohol, 2011. **46**(3): p. 278-82.
7. Jones, A.W., R.G. Hahn, and H.P. Stalberg, *Distribution of ethanol and water between plasma and whole blood; inter- and intra-individual variations after administration of ethanol by intravenous infusion.* Scand J Clin Lab Invest, 1990. **50**(7): p. 775-80.
8. Umulis, D.M., et al., *A physiologically based model for ethanol and acetaldehyde metabolism in human beings.* Alcohol, 2005. **35**(1): p. 3-12.
9. Lieber, C.S. and C.S. Abittan, *Pharmacology and metabolism of alcohol, including its metabolic effects and interactions with other drugs.* Clin Dermatol, 1999. **17**(4): p. 365-79.
10. Swift, R., *Direct measurement of alcohol and its metabolites.* Addiction, 2003. **98 Suppl 2**: p. 73-80.
11. Jones, A.W., *Excretion of alcohol in urine and diuresis in healthy men in relation to their age, the dose administered and the time after drinking.* Forensic Sci Int, 1990. **45**(3): p. 217-24.
12. Hoffman, P.L., J. Glanz, and B. Tabakoff, *Platelet adenylyl cyclase activity as a state or trait marker in alcohol dependence: results of the WHO/ISBRA Study on State and Trait Markers of Alcohol Use and Dependence.* Alcohol Clin Exp Res, 2002. **26**(7): p. 1078-87.
13. Schmitt, G., et al., *Ethyl glucuronide: an unusual ethanol metabolite in humans. Synthesis, analytical data, and determination in serum and urine.* J Anal Toxicol, 1995. **19**(2): p. 91-4.
14. Helander, A. and O. Beck, *Mass spectrometric identification of ethyl sulfate as an ethanol metabolite in humans.* Clin Chem, 2004. **50**(5): p. 936-7.
15. Dahl, H., et al., *Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide.* J Anal Toxicol, 2002. **26**(4): p. 201-4.
16. Wurst, F.M., et al., *Ethyl glucuronide--a marker of alcohol consumption and a relapse marker with clinical and forensic implications.* Alcohol Alcohol, 1999. **34**(1): p. 71-7.
17. Wurst, F.M., et al., *Ethyl sulphate: a direct ethanol metabolite reflecting recent alcohol consumption.* Addiction, 2006. **101**(2): p. 204-11.
18. Helander, A. and O. Beck, *Ethyl sulfate: a metabolite of ethanol in humans and a potential biomarker of acute alcohol intake.* J Anal Toxicol, 2005. **29**(5): p. 270-4.
19. Helander, A., et al., *Unreliable alcohol testing in a shipping safety programme.* Forensic Sci Int, 2009. **189**(1-3): p. e45-7.
20. Helander, A., et al., *Detection times for urinary ethyl glucuronide and ethyl sulfate in heavy drinkers during alcohol detoxification.* Alcohol Alcohol, 2009. **44**(1): p. 55-61.

21. Helander, A., et al., *Time course of ethanol-induced changes in serotonin metabolism.* Life Sci, 1993. **53**(10): p. 847-55.
22. Helander, A., et al., *Urinary excretion of 5-hydroxyindole-3-acetic acid and 5-hydroxytryptophol after oral loading with serotonin.* Life Sci, 1992. **50**(17): p. 1207-13.
23. Beck, O., et al., *5-hydroxytryptophol in the cerebrospinal fluid and urine of alcoholics and healthy subjects.* Naunyn Schmiedebergs Arch Pharmacol, 1982. **321**(4): p. 293-7.
24. Beck, O., et al., *Changes in serotonin metabolism during treatment with the aldehyde dehydrogenase inhibitors disulfiram and cyanamide.* Pharmacol Toxicol, 1995. **77**(5): p. 323-6.
25. Helander, A. and C.J. Eriksson, *Laboratory tests for acute alcohol consumption: results of the WHO/ISBRA Study on State and Trait Markers of Alcohol Use and Dependence.* Alcohol Clin Exp Res, 2002. **26**(7): p. 1070-7.
26. Beck, O. and A. Helander, *5-hydroxytryptophol as a marker for recent alcohol intake.* Addiction, 2003. **98 Suppl 2**: p. 63-72.
27. Doyle, K.M., et al., *Fatty acid ethyl esters in the blood as markers for ethanol intake.* JAMA, 1996. **276**(14): p. 1152-6.
28. Refaai, M.A., et al., *Liver and adipose tissue fatty acid ethyl esters obtained at autopsy are postmortem markers for premortem ethanol intake.* Clin Chem, 2002. **48**(1): p. 77-83.
29. Soderberg, B.L., et al., *Fatty acid ethyl esters. Ethanol metabolites that reflect ethanol intake.* Am J Clin Pathol, 2003. **119 Suppl**: p. S94-9.
30. Conigrave, K.M., et al., *Traditional markers of excessive alcohol use.* Addiction, 2003. **98 Suppl 2**: p. 31-43.
31. Puukka, K., et al., *Obesity and the clinical use of serum GGT activity as a marker of heavy drinking.* Scand J Clin Lab Invest, 2007. **67**(5): p. 480-8.
32. Niemela, O., *Biomarkers in alcoholism.* Clin Chim Acta, 2007. **377**(1-2): p. 39-49.
33. de Jong, G., J.P. van Dijk, and H.G. van Eijk, *The biology of transferrin.* Clin Chim Acta, 1990. **190**(1-2): p. 1-46.
34. Jeppsson, J.O., et al., *Toward standardization of carbohydrate-deficient transferrin (CDT) measurements: I. Analyte definition and proposal of a candidate reference method.* Clin Chem Lab Med, 2007. **45**(4): p. 558-62.
35. Alling, C., L. Gustavsson, and E. Anggard, *An abnormal phospholipid in rat organs after ethanol treatment.* FEBS Lett, 1983. **152**(1): p. 24-8.
36. Alling, C., et al., *Phosphatidylethanol formation in rat organs after ethanol treatment.* Biochim Biophys Acta, 1984. **793**(1): p. 119-22.
37. Aradottir, S., et al., *Phosphatidylethanol in human organs and blood: a study on autopsy material and influences by storage conditions.* Alcohol Clin Exp Res, 2004. **28**(11): p. 1718-23.
38. Aradottir, S., K. Moller, and C. Alling, *Phosphatidylethanol formation and degradation in human and rat blood.* Alcohol Alcohol, 2004. **39**(1): p. 8-13.
39. Varga, A., et al., *Normalization rate and cellular localization of phosphatidylethanol in whole blood from chronic alcoholics.* Clin Chim Acta, 2000. **299**(1-2): p. 141-50.
40. Gunnarsson, T., et al., *Determination of phosphatidylethanol in blood from alcoholic males using high-performance liquid chromatography and evaporative light scattering or electrospray mass spectrometric detection.* J Chromatogr B Biomed Sci Appl, 1998. **705**(2): p. 243-9.
41. Hansson, P., et al., *Blood phosphatidylethanol as a marker of alcohol abuse: levels in alcoholic males during withdrawal.* Alcohol Clin Exp Res, 1997. **21**(1): p. 108-10.
42. Varga, A., et al., *Phosphatidylethanol in blood as a marker of ethanol consumption in healthy volunteers: comparison with other markers.* Alcohol Clin Exp Res, 1998. **22**(8): p. 1832-7.
43. Stewart, S.H., et al., *Preliminary evaluation of phosphatidylethanol and alcohol consumption in patients with liver disease and hypertension.* Alcohol Alcohol, 2009. **44**(5): p. 464-7.

44. Aradottir, S., et al., *PHosphatidylethanol (PEth) concentrations in blood are correlated to reported alcohol intake in alcohol-dependent patients.* Alcohol Alcohol, 2006. **41**(4): p. 431-7.
45. Stewart, S.H., et al., *Phosphatidylethanol and alcohol consumption in reproductive age women.* Alcohol Clin Exp Res, 2010. **34**(3): p. 488-92.
46. Lundqvist, C., et al., *Agonist-stimulated and basal phosphatidylethanol formation in neutrophils from alcoholics.* Alcohol Clin Exp Res, 1994. **18**(3): p. 580-6.
47. Varga, A. and C. Alling, *Formation of phosphatidylethanol in vitro in red blood cells from healthy volunteers and chronic alcoholics.* J Lab Clin Med, 2002. **140**(2): p. 79-83.
48. Hanahan, D.J. and I.L. Chaikoff, *A new phospholipide-splitting enzyme specific for the ester linkage between the nitrogenous base and the phosphoric acid grouping.* J Biol Chem, 1947. **169**(3): p. 699-705.
49. Yang, S.F., S. Freer, and A.A. Benson, *Transphosphatidylation by phospholipase D.* J Biol Chem, 1967. **242**(3): p. 477-84.
50. Kobayashi, M. and J.N. Kanfer, *Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D.* J Neurochem, 1987. **48**(5): p. 1597-603.
51. Chalifa-Caspi, V., Y. Eli, and M. Liscovitch, *Kinetic analysis in mixed micelles of partially purified rat brain phospholipase D activity and its activation by phosphatidylinositol 4,5-bisphosphate.* Neurochem Res, 1998. **23**(5): p. 589-99.
52. Gustavsson, L., *ESBRA 1994 Award Lecture. Phosphatidylethanol formation: specific effects of ethanol mediated via phospholipase D.* Alcohol Alcohol, 1995. **30**(4): p. 391-406.
53. Wang, P., et al., *Existence of cytosolic phospholipase D. Identification and comparison with membrane-bound enzyme.* J Biol Chem, 1991. **266**(23): p. 14877-80.
54. Hammond, S.M., et al., *Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family.* J Biol Chem, 1995. **270**(50): p. 29640-3.
55. Hammond, S.M., et al., *Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-alpha.* J Biol Chem, 1997. **272**(6): p. 3860-8.
56. Steed, P.M., et al., *Characterization of human PLD2 and the analysis of PLD isoform splice variants.* FASEB J, 1998. **12**(13): p. 1309-17.
57. Selvy, P.E., et al., *Phospholipase d: enzymology, functionality, and chemical modulation.* Chem Rev, 2011. **111**(10): p. 6064-119.
58. Brown, H.A. and P.C. Sternweis, *Stimulation of phospholipase D by ADP-ribosylation factor.* Methods Enzymol, 1995. **257**: p. 313-24.
59. Pergola, C., et al., *Testosterone suppresses phospholipase D, causing sex differences in leukotriene biosynthesis in human monocytes.* FASEB J, 2011. **25**(10): p. 3377-87.
60. Lundqvist, C., et al., *Phosphatidylethanol formation and degradation in brains of acutely and repeatedly ethanol-treated rats.* Neurosci Lett, 1994. **179**(1-2): p. 127-31.
61. Wurst, F.M., et al., *Phosphatidylethanol: normalization during detoxification, gender aspects and correlation with other biomarkers and self-reports.* Addict Biol, 2010. **15**(1): p. 88-95.
62. Hodson, L., C.M. Skeaff, and B.A. Fielding, *Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake.* Prog Lipid Res, 2008. **47**(5): p. 348-80.
63. Engelmann, B., et al., *Molecular species of membrane phospholipids containing arachidonic acid and linoleic acid contribute to the interindividual variability of red blood cell Na(+)-Li+ countertransport: in vivo and in vitro evidence.* J Membr Biol, 1993. **133**(2): p. 99-106.
64. Holbrook, P.G., et al., *Molecular species analysis of a product of phospholipase D activation. Phosphatidylethanol is formed from phosphatidylcholine in*

phorbol ester- and bradykinin-stimulated PC12 cells. J Biol Chem, 1992. **267**(24): p. 16834-40.
65. Aradottir, S. and B.L. Olsson, *Methodological modifications on quantification of phosphatidylethanol in blood from humans abusing alcohol, using high-performance liquid chromatography and evaporative light scattering detection.* BMC Biochem, 2005. **6**: p. 18.
66. Varga, A. and S. Nilsson, *Nonaqueous capillary electrophoresis for analysis of the ethanol consumption biomarker phosphatidylethanol.* Electrophoresis, 2008. **29**(8): p. 1667-71.
67. Nissinen, A.E., et al., *Immunological detection of in vitro formed phosphatidylethanol--an alcohol biomarker--with monoclonal antibodies.* Alcohol Clin Exp Res, 2008. **32**(6): p. 921-8.
68. Nissinen, A.E., et al., *Low plasma antibodies specific for phosphatidylethanol in alcohol abusers and patients with alcoholic pancreatitis.* Addict Biol, 2011.
69. Tolonen, A., et al., *A method for determination of phosphatidylethanol from high density lipoproteins by reversed-phase HPLC with TOF-MS detection.* Anal Biochem, 2005. **341**(1): p. 83-8.
70. Radin, N.S., *Extraction of tissue lipids with a solvent of low toxicity.* Methods Enzymol, 1981. **72**: p. 5-7.
71. Aradottir, S., et al., *Phosphatidylethanol (PEth) concentrations in blood are correlated to reported alcohol intake in alcohol-dependent patients.* Alcohol Alcohol., 2006. **41**(4): p. 431-7.
72. Helander, A., A. Husa, and J.-O. Jeppsson, *Improved HPLC method for carbohydrate-deficient transferrin in serum.* Clin Chem, 2003. **49**: p. 1881-90.
73. Bergström, J.P. and A. Helander, *Influence of alcohol use, ethnicity, age, gender, BMI and smoking on the serum transferrin glycoform pattern: implications for use of carbohydrate-deficient transferrin (CDT) as alcohol biomarker.* Clin Chim Acta, 2008. **388**(1-2): p. 59-67.
74. Zheng, Y., O. Beck, and A. Helander, *Method development for routine liquid chromatography-mass spectrometry measurement of the alcohol biomarker phosphatidylethanol (PEth) in blood.* Clin Chim Acta, 2011. **412**(15-16): p. 1428-35.
75. Arndt, T., B.B. van der Meijden, and J.P. Wielders, *Atypical serum transferrin isoform distribution in liver cirrhosis studied by HPLC, capillary electrophoresis and transferrin genotyping.* Clin Chim Acta, 2008. **394**(1-2): p. 42-6.
76. Kenan, N., et al., *Changes in transferrin glycosylation during pregnancy may lead to false-positive carbohydrate-deficient transferrin (CDT) results in testing for riskful alcohol consumption.* Clin Chim Acta, 2011. **412**(1-2): p. 129-33.
77. Gnann, H., et al., *Identification of 48 homologues of phosphatidylethanol in blood by LC-ESI-MS/MS.* Anal Bioanal Chem, 2010. **396**(7): p. 2415-23.
78. Crowe, F.L., et al., *Serum phospholipid n 3 long-chain polyunsaturated fatty acids and physical and mental health in a population-based survey of New Zealand adolescents and adults.* Am J Clin Nutr, 2007. **86**(5): p. 1278-85.
79. Nalesso, A., et al., *Analysis of the alcohol biomarker phosphatidylethanol by NACE with on-line ESI-MS.* Electrophoresis, 2010. **31**(7): p. 1227-33.
80. Nalesso, A., et al., *Quantitative profiling of phosphatidylethanol molecular species in human blood by liquid chromatography high resolution mass spectrometry.* J Chromatogr A, 2011. **1218**(46): p. 8423-31.
81. Faller, A., et al., *LC-MS/MS analysis of phosphatidylethanol in dried blood spots versus conventional blood specimens.* Anal Bioanal Chem, 2011. **401**(4): p. 1163-6.
82. Gnann, H., et al., *Selective detection of phosphatidylethanol homologues in blood as biomarkers for alcohol consumption by LC-ESI-MS/MS.* J Mass Spectrom, 2009. **44**(9): p. 1293-9.