The use of phosphatidylethanol (PEth) as a sensitive biomarker for alcohol consumption in clinical routine analysis

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Abstract

**Background**: Phosphatidylethanol (PEth) is a direct ethanol metabolite. It is an abnormal cellular membrane phospholipids that is formed only in the presence of ethanol by the enzyme phospholipase D. PEth can be detected in blood for more than 2-3 weeks after sustained ethanol intake.

**Aims**: Our main aims were (i) to analyse PEth in clinical routine samples by UPLC-MS/MS, (ii) to evaluate the use of PEth as an alcohol biomarker and (iii) to evaluate the use of PEth in different diagnostic laboratories.

**Methods**: PEth was analysed in blood samples (n = 96) that were collected for clinical routine analysis, including 4 calibrators, 4 negative controls, 4 positive controls and a blank. PEth identification was based on ESI-tandem mass spectrometry (MS/MS) analysis of the different ions in a single gradient run with 2 µL injection volume. The different cut-off and internal control values were the criterion measure for marker sensitivity.

**Results**: Using an internal calibration curve, the concentrations of 91 different PEth samples were estimated. These samples were collected from different clinics sent to the laboratory. Of these samples 42 (43.75) % had a PEth concentration below < 0, 05 µmol/L which is the lower limit of alcohol intake. There are no significant differences when comparing PEth result in eight different laboratories. This signals that the method is good and the PEth concentrations from different laboratories are comparable.

**Conclusion**: PEth is a useful test for detecting alcohol consumption in individuals under detoxification program. The present results indicate that, LC-MS/MS method is a highly sensitive and selective method that enables a detection of extremely low levels of PEth.

**Keywords**: PEth, tandem mass spectrometry (MS/MS) and electron spray ionization.
List of abbreviations

ACN: Acetonitrile
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
CDT: Carbohydrate-deficient transferrin
DBS: Dried blood spot
ESLD: Evaporative light scattering detector
ESI: Electron spray ionization
EtS: Ethyl sulphate
EtG: Ethyl glucuronide
EDTA: Ethylenediaminetetraacetic acid
EP: European pharmacopoeia
FAEE: Fatty acid ethyl esters
FDA: Food and drug administration
GGT: Gamma-glutamyltransferase
5-HTOL: 5-hydroxytryptophol
HPLC: High performance liquid chromatography
IQC: Internal quality control
LOQ: Lower limit of quantification
LC-MS: Liquid chromatography mass spectrometry
MCV: Mean corpuscular volume
MF: Match factor
NHP: Nation Harmonisation Programme
PEth 16:0/18:1: Phosphatidylethanol with the fatty acid chains 16:0/18:1
D31-PEth 16:0/18:1: Deuterated Phosphatidylethanol with the fatty acid chains 16:0/18:1
PC: Phosphatidylcholine
PLD: Phospholipase D
RD: Reference database
RT: Retention time
SIM: Selected ion monitoring
TLC: Thin-layer chromatography
UPLC-MS/MS: Ultra-performance liquid chromatography tandem mass spectrometry
UOQ: Upper limit of quantification
INTRODUCTION

Alcohol is the world’s top three priority areas in public health. Alcohol use and dependence are very prevalent disorders that can lead to a multitude of medical, economic and social issues. In our social cultures, the habit of drinking alcohol is deeply rooted and over consumption is not only linked to liver disease, but is also associated to cancer and cardiovascular disease. Moreover, alcohol misuse is known to be the major causes of road accidents, premature death, health disorders, violence, damage to personal property and criminal activity [1, 2].

In order to assess a person’s drinking behaviour as healthy or unhealthy, objective clinical guidelines and diagnostic protocols are greatly needed in most research and clinical treatment areas [3, 4]. A number of different approaches have been established during the past decades with limitations in the forensic setting to characterize the individual’s differences, based on their drinking patterns [5, 6]. However, the limited diagnostic efficiency of reported biomarkers and the difficulty in assessing alcohol-drinking behaviour from an objective point of view, have led to the introduction, evaluation and validation of different reliable biological markers of chronic and excessive alcohol drinkers in different clinical evaluations.

Increasingly, alcohol biomarkers are used as objective measures of the performance of alcohol abuse treatment and to track the progress of diseases related to alcohol abuse in most clinical settings. These biological markers can be broadly divided into two categories, namely indirect and direct biomarkers. Indirect biomarkers detect the effects of alcohol on organ systems and body chemistry, and generally comprise markers of toxic effects including gamma-glutamyltransferase (GGT), mean corpuscular volume (MCV), aspartate aminotransferase (AST), alanine aminotransferase (ALT), 5-hydroxytryptophol (5-HTOL), plasma sialic-acid index of apolipoprotein J (SIJ), and the widely used standard method, carbohydrate-deficient transferrin (CDT)[7,8,9]. Direct markers include blood ethanol and its derivatives such as fatty acid ethyl esters (FAEE), ethylglucuronide (EtG), ethyl sulphate (EtS), and phosphatidylethanol (PEth) [10].

Currently, clinically established biomarkers in blood that indicate alcohol use and abuse are GGT, MCV and CDT. Although widely used, none of these established markers is 100%
specific and sensitive enough to detect the degree of alcohol consumption [11]. Combinations of tests such as, GGT and CDT may show an increase degree of diagnostic accuracy, but also, there are clinical variability of ethanol and its derivatives in half-lives, thus requiring further investigations, especially in situations when alcohol over-consumption is to be identified [12]. In light of this shortcoming variability, there is a crucial need for the development of more sensitive and specific marker of alcohol use and abuse.

Among these biomarkers, PEth in blood has recently attracted international attention over the last decade as a potential biomarker of alcohol use and abuse. PEth is a direct ethanol metabolite that is formed only in the presence of alcohol and phosphatidylcholine (PC) by the action of phospholipase D (PLD) [13]. Normally, PLD forms phosphatidic acid using water as substrate, but when ethanol is present, PLD has a higher affinity for the alcohol and forms PEth. PEth was first discovered in mammalian organs and tissues in 1983, being detected in the kidney, brain, skeletal muscle, heart and liver of rats chronically exposed to ethanol [14]. PEth has been proven to be a sensitive biomarker (99 %) for alcohol than CDT (sensitivity of 77 %) and GGT [15, 16, 17].

![PEth-16:0/18:2](image1.png)  
![PEth-16:0/18:1*](image2.png)

**Fig. 1. An illustration of two out of many PEth molecular species.** Nomenclature for fatty acids corresponds to the number of carbon atoms) :( numbers of double bonds). * is the commercially available material, Helander & Zheng, 2009 [21].

Historically, the first technique used for the measurement of total PEth in whole blood from alcoholic patients was by thin-layer chromatography (TLC) and was later replaced by high-performance liquid chromatography with evaporative light-scattering detector (ELSD) [15,18]. More recently, due to the increasing importance of reliability, sensitivity, selectivity and specificity of alcohol biomarkers in clinical routine evaluations, a new method for
determination of molecular species of PEth by LC-MS/MS in human whole blood after liquid-liquid extraction from cell membrane with a shorter analysis time of 4 min was successfully developed [21]. Other studies, conducted on rats chronically exposed to ethanol, demonstrated that PEth is not a single molecule, but a comprised group of glycerophospholipid homologous with a common phosphoethanol head group onto which 2 fatty acid moieties attached together (19). And later, the development of LC-MS/MS method that has led to a revolution in diagnostic testing of clinical routine PEth samples in whole blood (20). This study demonstrated an overall good correlation for PEth 16:0/18:1 and PEth 16:0/18:2, see the molecular structures for these compounds in Fig.1. Both species accounted for approximately 60% of the total concentration in blood from heavy alcohol drinkers [20] as previously documented [19]. Of these, PEth 16:0/18:1 is now the principal and most recommended form for PEth quantification for clinical routine evaluation. The advantage of using the LC-MS/MS with an electron spray ionization (ESI) compared to ELSD is due to it greater sample throughput [15, 18]. Another great advantage of using the LC-ESI-MS/MS is due to its lower detection limit of PEth in samples, which allows for the determination of previously consumed ethanol in patients whole blood sample.

LC-MS/MS method gives a high analytical sensitivity with high analytical specificity, often allowing relatively short chromatography run-times, easier workflows, characterized by small particle sizes less than 2 um, and higher throughput pressure of 100MPa than 40MPa in conventional HPLC with larger particle size of 5 um, which thus makes this separation technique so efficient and very exciting, improved resolution for complex mixtures of components, reduces operational cost, as well as the possibilities of analysing a good number of samples at a single run time.

In other clinical studies conducted on chronic heavy drinkers, PEth was found to decrease over time, with a half-life of about 3-5 days or 6-9 days in some studies, and remained detectable in the blood up to 28 days after alcohol withdrawal; therefore it is a promising new biomarker of alcohol consumption [22]. Additionally, it has been shown that, sex, age and body mass index do not influence the normalized rate of blood PEth “the adjustment of PEth quantification data for different effects which arise from variation in the method rather than from biological differences between PEth samples” [22]. PEth has been found to have a sensitivity of between 94.5% and 100%, and specificity of 100% [15, 22]. Despite the high performance of PEth over other biomarkers, the current UPLC-MS/MS method for
quantification and evaluation of PEth in whole blood samples for clinical routine use is still in its early age of clinical verification and hence, requiring new experimental data from earlier procedures to guide further development and collaborations between diagnostic laboratories.

In Sweden, the lowest limit cut-off value of reporting PEth is set to 0.05 µmol/L-PEth-16:0/18:1 and the upper value is 0.30 µmol/L-PEth-16:0/18:1, which is currently considered to represent an over consumption rate of alcohol use with respect to moderate i.e. being the lower limit cut-off value [23].

**The masspectrometry method**

Masspectrometry (MS) is a very selective method that analyses the molecular ion based on the weight and charge (m/z). The quadrupole filters away unwanted analytes and let the specific molecular ion through to be detected. There are different masspectrometry. In single MS only the molecular ion is detected. In tandem MS (MS/MS) the molecular ion is fragmented and the fragments are detected. Figure 2 illustrates single MS and two of many more MS/MS mechanism that are possible to perform.

![Masspectrometry Diagram](image)

**Fig. 2. An illustration of tandem mass spectrometry (MS/MS) in Time Modes.** Classic modes performed by ion-traps, ions of all intended molecules in samples are first trapped. All but the intended ion which is to be fragmented is ejected from the trap. The trapped ions are later excited by collision, and therefore are induced to fragment in the presence of Helium gas. In the next stage, the sequential fragments are ejected onto a detector to generate a mass spectrum of the intended molecular fragments. This trapping and fragmentation process is unique and may be repeated several times in order to obtain the specific fragment.
Aims

The aims of the present study were to:

(i) To analyse PEth in clinical routine samples by using UPLC-MS/MS.
(ii) To evaluate the use of PEth as an alcohol biomarker.
(iii) To evaluate PEth results from different laboratories in Sweden.

Materials and Methods

Chemicals and Solutions

The solvents used during the solid-phase extraction and UPLC analysis were methanol (MeOH), 2-propanol, and acetonitrile (ACN) purchased from (Sigma-Aldrich, Germany); all were of LC-MS-grade. Acetic acid and ammonium acetate (0.543M) CAS Nr: 631-61-8 were purchased from (Sigma-Aldrich, Germany). A deuterated phosphatidylethanol, PEth-D-31, 160D31-181PEtOH-11 from Avanti Polar Lipids. PEth-16:0/18:1 reference material containing 1 palmitic and 1 oleic acid (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol. from Biomol Research Laboratories. All other chemicals were of analytical or UPLC grade. Deionized sterile filtered water was purchased from Millipore Milli-Q plus water purification system and was regularly checked for conductivity.

Reference material

Stock solutions of PEth reference materials (PEth-D31 1.33 mmol/L (150 μL) and 2-propanol (200 mL) were prepared in a 1.5 solution of 2 mmol/L ammonium acetate and acetonitrile and stored at -20 ºC until further use. Under these conditions, the solutions were stable for at least 6 months [20].
**Blood samples**

The blood specimens used for the evaluation study were surplus volumes from the clinical sample pool sent to the Alcohol Laboratory (Unilabs AB, Eskilstuna, Sweden) for testing of alcohol consumption. For internal quality control (IQC), additional samples \( n = 4 \) of concentration 0.05 \( \mu \text{mol/L} \) were used as the lower controls and samples \( n = 4 \) of concentration 1.00 \( \mu \text{mol/L} \) were used as the upper controls. These samples were obtained from previously screened patients undergoing alcohol detoxification program PEth results. The main role of the IQC is to monitor the day-to-day precision, variability, accuracy and validation. For external quality assessment (EQA), samples \( n = 3 \) with concentrations of 0.27, 0.0 & 1.2 \( \mu \text{mol/L} \) were obtained from EQUALIS.

**Deuterated internal standard**

A deuterated phosphatidylethanol internal standard (IS) PEth-D31, was used in the evaluation of PEth. The main use of the IS in the procedure was to compensate for losses during sample preparation and variable detection sensitivity of the ESL-MS/MS system. PEth-D31 IS is the same chemical as the intended measuring analyte compound “PEth-16:0/18:1. A deuterated (IS) have a similar chromatographic retention time, extraction recovery and ionization response in ESI mass spectrometry as PEth-16:0/18:1. PEth-D31 coe-lutes exactly with PEth-16:0/18:1 and helps to effectively eliminate variations in sample preparation, injection, ionization, errors due to non-linearities such as matrix effects, instrument performance at different intervals, regardless of the environmental changes such as temperature.

**Preparation of calibration standards and quality control**

Four calibration standards stock solutions of concentrations (0, 01, 0, 05, 1 and \( \mu \text{mol/L} \)) were spiked to PEth negative blood samples. The quality control samples were made by pooling from blood samples that was positive in PEth.
Sample collection

Venous blood samples were collected into ethylenediaminetetraacetic acid EDTA tubes of 3 mL and stored at +4 °C for a few hours before analysing and the samples were not centrifuged. Blood samples for PETH analysis have been proven to be stable for 24 h at room temperature and for 3 weeks at +4 °C [25]. If whole blood samples are not used immediately, they should be frozen in plastic tubes in a -80 °C refrigerator blood samples, to avoid any in vitro PETH formation [15, 17, 18].

Sample preparation

Extraction of phosphatidylethanol from blood

Prior to extraction, (EDTA) whole blood samples were removed from the +4 °C storage unit, scanned and computerized to create a database of patients for results reporting and feedback before vortexing for 10 minutes and thereafter, PETH was extracted from (EDTA) whole blood with isopropanol, containing (phosphatidylethanol -D31-16:/0/18:1(PETH-D31) reference substance, as the internal standard (IS) and acetonitrile (ACN), (liquid-liquid extraction). After vortexing, 100 μL of whole blood was pipetted using a special robot automation system (Tecan Freedom EVO 100) into micro tubes, and 200 μL IS mixture was added and briefly vortexed. The next step was the addition of 800 μL of ACN followed by vortexing. The samples were finally centrifuged at 2000 g for 5 min at 4 °C. After centrifugation, 800 μL of the cleared supernatants were transferred to a clean 96 well.

Ultra performance liquid chromatography (UPLC)

The Acquity, Ultra Performance LC-system (Waters Xevo TQ) consisting of a degasser, a binary gradient pump, an auto injection thermostat (10 °C) and a column oven (15°C) were used for PETH-16:0/18:1 quantification. Eluents used were H2O/ACN/ ammonium acetate (0,543 Mol/L (78.5:20:1.5) mL by Vol (A) and by MeOH /ACN (80:20) mL by Vol (B) stored at room temperature for a maximum period of six months. The flow-rate was 0.6 mL/min. Sample volumes of 2 μL were injected. Gradient conditions were as follows: 0-0.2 min, linear from 13% A to 87% B; 0.2-2.0 min, isocratic 100% B; 0.2-2.5 min, isocratic 100% B; 2.5-3.00 min, linear from 13% A to 87% B.
Statistical analysis

Data obtained from the MassLynx (version 4) analysis software was analyzed using a one way analysis of variance (ANOVA) test embedded in Graphpad Prism 6 statistical software. A probability of $P \leq 0.01$ was considered to be significant.

Results

The parameters and the transitions of the electron spray ionisation tandem mass spectrometry were consistent with previous reports (21). The precursor’s ions for the analytes, PEth and the internal standard PEth-D31, were the deprotonated molecular weight ions m/z 702.0 and m/z 733.0 respectively. Two different multiple reaction monitored (MRM) transition fragments 281.3 and 255.3 were observed. The m/z 702 -255 MRM transition was used to record the chromatogram of PEth (Fig. 3).

An MS/MS chromatogram

A typical chromatogram obtained from an UPLC-MS/MS system is presented below (see fig.3). The fragments 281.3 m/z and 255.3 m/z is monitored for PEth-16:0/18:1 and 281.3 m/z is monitored for PEth-D31.

Fig. 3. LC-MS/MS- chromatogram of PEth -16:0/18:1 molecular specie in blood.
Calculation of PEth-16:0/18:1 in samples using calibration curve

The calibration curve is based on four different concentrations that are known see table 1. These concentrations are then plotted in a diagram to obtain a linear equation see fig. 4.

Tab.1. LC-MS/MS calibration curve

<table>
<thead>
<tr>
<th>PEth µmol/L</th>
<th>Int. std. µmol/L</th>
<th>Mx/Mis</th>
<th>Ax</th>
<th>Ais</th>
<th>Ax/Ais</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>1</td>
<td>0.01</td>
<td>28</td>
<td>1845</td>
<td>0.0151</td>
</tr>
<tr>
<td>0.05</td>
<td>1</td>
<td>0.05</td>
<td>187</td>
<td>1868</td>
<td>0.1001</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4301</td>
<td>1988</td>
<td>2.163</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>5</td>
<td>22261</td>
<td>1978</td>
<td>11.25</td>
</tr>
</tbody>
</table>

**Footnote:** Internal standard and PEth concentration used were in µmol/L. Ax; peak area of PEth in samples, Ais; peak area of the internal standard in samples. Both Mx/Mis and Ax/Ais are respective constant without a standards unit.

We observed a strong correlation of $r^2$ value (or the Pearson Coefficient of Determination) = 0.9999 and $Y = 2.2466x$ (Fig.3). This is an indicator of how well our data fits the standard curve. All the 4 points of concentrations (0.01, 0.05, 1 & 5) µmol/L lie exactly on a straight line showing a good linear relation. Ideally, since $r^2$ is closer to 1, the more likely that our data points X and Y (Tab.1, Fig. 4) are actual solutions to the equation. This indicates how well our data fits the intended analytical model described by the equation $Y = Kx + Mo$, where Y is the instrument response, K represents the sensitivity, and Mo is a background constant.

![Fig. 4. LC-MS/MS calibration curve for PEth with internal standard](image)

*Fig. 4. LC-MS/MS calibration curve of PEth-16:0/18:1 with PEth D-31 as an internal standard, showing a linear response throughout the range of expected concentration. Ax: peak area of PEth in samples, Ais: peak area of the internal standard in samples, Mx: concentration of PEth in samples and Mis: concentration of internal standard in samples. The above variables could be summarized using the standard derived equation below, where Fx is the response factor or the slope.*
Examination of PEth 16:0/18:1 by LC-MS/MS

The PEth molecular specie included in this evaluation was (PEth-16:0/18:1) and a deuterated Phosphatidylethanol with the fatty acid chains 16:0/ 18:1 (D31-PEth) an internal standard (IS) of PEth-16:0/18:1 “reference substance” was added to the unknown patient samples during the pre-treatment procedure to provide accurate quantification during the whole quantification process. Following the national established protocol [21], during phospholipids extraction from human erythrocytes, saturated fatty acids (e.g., 16:0, palmitic acid) are mainly localised in the sn-1 position and the unsaturated fatty acids (e.g., 18:1, linoleic acid) mainly in the sn-2 position.

The principal ions product of PEth, as identified by MS/MS, is compatible with the fatty acid chains [21]. The identity of the major ions products was confirmed by the simultaneous analysis of PEth reference material, where PEth-16:0/18:1 produced 1 major product ion each (m/z 255.5 for 16:0 fatty acid and 281.5 for 18:1 fatty acid, See Fig.2 & Fig.3 for more detailed illustration of different ions fragmentation by a tandem mass spectrometry (MS/MS). The identification of PEth-16:0/18:1 in whole blood samples was based on selected ion monitoring (SIM) analysis of the distinctive mass ratios for the fatty acid chains to the parent compound, where similar ion ratios were produced as compared with the reference material [21].

Fig. 5. Structures of different PEth species and phosphatidylpropanol. The tentative structures of the molecular species of the PEth and PProp (internal standard (IS) measured by the LC-SM method. Nomenclature for fatty acids corresponds to the number of carbon atoms): (numbers of double bonds). * is the commercially available material, Helander & Zheng, 2009 [21].
UPLC-MS/MS profiling

We evaluated whole blood samples from patients following a detoxification program whose clinical samples were sent to the Alcohol Laboratory (Unilabs AB, Eskilstuna, Sweden) for testing of alcohol consumption. The results of the measurement range and the UPLC-MS/MS quantification analysis are summarized by a histogram distribution (Fig. 6 & Fig.7). Following our statistical calculations, there were no statistically significant differences between group means as determined by one-way ANOVA (Fig.8). For the internal control samples, the evaluation was repeated thrice, with the same negative pool of blood samples with reproducible results.

Fig. 6. Profile of PEth-16:0/18:1 in blood from alcohol patients including four standards calibration curve samples and a blank sample. A histogram showing the frequency distribution of PEth-16:0/18:1 in whole blood samples collected from 91 patients undergoing detoxification treatment for alcohol-related problems. As shown above, n = 13 (13.54%) of samples 0.0 μmol/L concentration indicates zero alcohol consumption, n = 29 (30.21%) of samples < 0.05 μmol/L concentration indicates a sporadic pattern of alcohol consumption, n = 18 (18.75 %) of samples 0.05-0.30 μmol/L concentration indicates moderate alcohol consumption and n = 36 (37.5% of samples > 0.30 μmol/L concentration indicates heavy or regular alcohol consumption. All samples were evaluated using the LC-ESI-MS/MS- time mode and concentrations were grouped following the national cut-off value.
Fig. 7. Comparison of PEth concentrations in patient’s whole blood samples by LC-MS/MS over 8 diagnostic laboratories undergoing PEth. (A) Variation of three different PEth concentrations (0.27, 0.0 & 1.2 µmol/L). (B) Mixture of whole blood samples from non-drinkers with PEth-16:0/18:1 reference substance (0, 30 µmol/L). (C) Whole blood from non-alcohol drinkers and (D) Mixture of patient’s whole blood samples containing phosphatidylethanol (1.12 µmol/L). Samples results from the eight laboratories were single observations that were included in the statistical analysis. Also, there were no statistically significant differences between group means as determined by one-way ANOVA of (F =1.61, P = 0.3315).

Fig. 8. Method development showing different variation separation techniques used for PEth analysis. LC-MS/MS method has seen unprecedented growth during the last decade in clinical PEth evaluation in Sweden from one laboratory in 2010 to over 8 different laboratories in 2014, in clinical routine analysis for PEth and other associated drugs.
Growth of clinical LC-MS/MS Use of PEth in Sweden

It has been shown previously that the majority of published clinical data were based on the total PEth concentration by HPLC-ESL-detection method [17, 20]. Nowadays, this method is considered unsuitable and no longer used. Thanks to a faster, more reliable LC-MS/MS method that has seen unprecedented growth during the last decade in clinical PEth evaluation in Sweden from one laboratory in 2010 to over 8 different laboratories in 2014, in clinical routine analysis for PEth (Fig.8) (23). Today, all laboratory measures PEth 16:0/18:1 by LC-MS/MS as seen on (Fig.8), which is the most distinctive specie [20].

Discussion

In this study, we demonstrated the feasibility of using an UPLC-MSMS analytical technique to quantify and analyze PEth concentration in whole blood. We observed the same results patterns across 8 different PEth diagnostic laboratories. This was because of the used of a common standard national diagnosing protocol for PEth-16:0/18:1” as shown above in (Fig. 7A, 7B, 7C, & 7D).

We were able to evaluate a number of clinical samples that can be analysed within a short time. This report is the first to evaluate and compare PEth clinical samples between different laboratories in Sweden. After PEth isolation from whole blood samples, a number of different PEth concentrations were obtained using MSMS (Fig. 6).

Historically, the first technique used for the measurement of total PEth in blood from alcoholic patients was by thin-layer chromatography (TLC) and was later replaced by high-performance liquid chromatography with evaporative light-scattering detector (HPLC-ELSD) [15,18]. Today PEth is analysed with LC-MS/MS, a sensitive technique that offers higher resolution, less solvent consumption and shorter analysis time than the previously used HPLC-ELSD technique.
Advantages of PEth over other alcohol biomarkers

PEth is a biomarker that can only be formed when alcohol is present “hence high specificity”. Moreover, PEth has a high sensitivity for distinguishing alcohol misused individuals. Also, PEth concentrations in blood have been shown to correlate to reported alcohol intake in alcohol-dependent patients [15].

For clinical use of PEth as alcohol biomarker, previous studies have used cut-off values for total PEth in the range of approximately 0.2-0.7 μmol/L, depending on the lower limit of quantification (LOQ) of the HPLC used method at the time [21]. In Sweden dating as far back as 2013-09-16 until present, 0. 30 μmol/L is the currently used as the routine clinical threshold value (Tab. 1). Based on previous studies [21], the lower limit of PEth measurement by LC-MS/MS method was set to 0. 02 μmol/L for each PEth species. However, these cut-off values are no longer in use and have been replaced with 0. 05-0.3 μmol/L for single most significant specie (PEth-16:0/18:1).

Based on Fig. 8, we could therefore hypothetically correlate our PEth results evaluation’s cut-off values in support of previous studies, detectable by PEth analysis which was approximately below 40 g/day [15], were detectable when the LOQ was reduced to approximately 0. 05μmol/L. Whereas amounts above 50 g/day [25], were detected when the LOQ was increased to approximately 0. 30 μmol/L “over consumption rate (regular intake)” of single PEth-16:0/18:1) by LC-MSMS. Results over 0. 30μmol/L are stipulated to be over consumption rates beyond the upper limit of quantification (UOQ) (Fig. 4).

The advantages of using PEth over others alcohol biomarker (e.g., CDT) is the high sensitivity between 94.5% and 100%, and specificity of 100% [15, 22]. Moreover, it has been shown that gender, sex, age and body mass index do not influence the normalized rate of blood PEth concentration [22]. Others studies have shown that advanced gestational and age increases serum carbohydrate -deficient transferrin levels in abstinent pregnant women due to changes of transferrin glycosylation, leading to false-positive results in testing for risky drinking consumption [26, 27].

In other clinical studies conducted on chronic heavy drinkers, PEth was found to decrease over time, with a half-life of about 3-5 days, and remained detectable in the blood up to 28
days after alcohol withdrawal; therefore it is a promising new biomarker of alcohol consumption [22].

A main drawback with PEth is the risk for in vitro formation or “post” sampling production containing ethanol, especially when stored at -20 °C for a long period, as previously demonstrated [24,28], which could create false-positive results. This risk could occur in purely alcohol abstinent patient’s samples before whole blood withdrawal and in vitro ethanol formation between time of evaluation due to microbial action.

Other factors which could have influenced our results could be due to a number of errors that can occur when preparing the standard curve. The consequences of inaccurate pipeting technique of the standard calibration dilutions could lead to the production of an inaccurate standard curve, resulting in lower or higher amplification efficiency scores in the data generated. A good example of variation in pipetting was observed in one out of the eight laboratories “lab 4” (Fig. 7C). Moreover, pipetting accuracy and reproducibility are the most important considerations in routine clinical testing of PEth samples. This accuracy and reproducibility can be enhanced by regular verification that the pipette in use meets the “manufacture’s specification” a check which can be achieved by using a standard performance scale balance. Others factors like washing, incubation time and temperature may also cause differences for the evaluation of PEth results.

Also, the skill of the laboratory technician plays a very important role in the precision and accuracy of the pipette. The choice of the pipetting technique (e.g., forward mode versus reverse mode) could also lead to variability in pipetting volumes as well as differences in data generated between duplicated samples as shown in “lab 4” (Fig. 7C). Proper training on both techniques can help reduce false positive results by ensuring good pipette precisions and reproducibility across laboratory technicians. In addition, we recommend a periodic pipette calibration and to always inspect the pipette and tips for correct seal to ensure a consistent result of the standard curve.
Quality controls

The main aim of all clinical laboratory services strive to ensure that the right results are obtained in patients samples analysis. Decisions on patient’s management on medications are made on the basis of these precise and accurate results. For internal quality control (IQC), a total 4 positive PEth blood concentrations, 4 negative PEth concentrations, blank and 4 calibrators with different concentrations (Tab.1 & Fig.3) sample were used during the evaluation. In analytical chemistry laboratory, quality control forms the basic foundation of the quality system. Quality control aimed to assure the reliability of the results for the specific analyst and also, the ability to ensure that consistency is maintained in the preceding evaluations following variation in environmental conditions.

Advantages of internal standardization

Internal standards calibrations in clinical chemistry involves the comparison of the measuring instrument responses from the intended target compounds in the sample to the response of the internal standard (IS) such as PEth-D31. The IS was added to the samples during the preparation process or sample extract before the injection stage. The ratio is termed the response factor (Fx), indicating that the target compound response is obtained relative to that of the internal standard. [31]. PEth-D31 was used to improve the precision and accuracy of the mass spectrometry. Ideally, IS behaves similarly to the analyte but to an extern, provide a distinguished signal from that of the analyte “PEth” (Fig.4). In the absence of the IS in mass spectroscopy analysis, fluctuations in temperature could affect the analyte signal by changing the degree of thermal excitation and atoms ionization. A constant concentration of the IS must be the same in all solutions indicating a good precision of the analytical instrument. It is highly recommended to monitor the IS area of PEth-D1 for all batches during PEth evaluation. A systematic variation of the IS area during several batches can indicate a hardware related problems such as a contaminated column or ion sources from matrix effects.
Limitations of PEth analysis method

Analysis of body fluids in clinical chemistry laboratories is generally subjected to different interferences that affect the analytical accuracy. The matrix interference effect is differences caused by sampling properties other than the specific intended sample to be measured and may include mechanistic, physiochemical and analytical interferences as well as substances isoforms. The major sources of endogenous interference are hemolysis, lipemia, bilirubinemia and paraproteinemia. These endogenous sources generally limit clinical routine PEth evaluations.

Conclusion

In this present study, we used an analytical chromatographic separation approach consisting of UPLC-MS/MS to quantify and analyse PEth in whole blood from patient samples sent to the laboratory for routine analysis. By this sensitive and specific separation technique, we were able to analyse a good number of PEth sample profile of patients, including the different internal controls. The different PEth profiles were reported based on the national cut-off stipulated values. PEth is a useful test for detecting alcohol use in patients. Also, UPLC-MS/MS has the potential to support routine clinical PEth evaluations. The selectivity and sensitivity of this analytical method enables the detection of extremely low-level of PEth concentrations in samples, showing an advantage over the traditional HPLC with UV-detection.

A further understanding of different cut-off values of social drinkers and the use of other significant PEth homologous (PEth-16:0/18:2) biomarker will contribute to new knowledge in alcohol detoxification biology which could help in building a better foundation for the development of new diagnostic protocols and methods for PEth analysis.
**Future perspective**

A future perspective of this study would be to reduce the time of sample preparation before LC-MS/MS analysis. To achieve this, we will use an automatic embedded vibrator on the Tecan technologies. This process could as well limit hand to hand sample contacts by laboratory personals, as well as the total time between vortexing and pipetting EDTA whole blood samples.

Stability and in-vitro formation of PEth (especially in samples containing ethanol) are further drawbacks of PEth to be used as a marker for detection of regular alcohol users. An alternative sample withdrawal and storage method to avoid this problem could hence be achieved by the use of dried blood spots (DBS) for clinical routine LC-MS/MS method for PEth analysis. DBS methods have recently been used and established as a valuable tool in therapeutic drug monitoring [29, 30].

DBS could offer advantages over traditional sampling techniques including: storage, sensitive, accurate, precise analysis, reduces human intervention during sampling collection, resulting in reduced stress when compared to whole blood, reduced laboratory cost, test substances volumes, reduces volumes enables serial sampling of EDTA tubes during pre and post analysis, simplified procedures (collection and laboratory) storage and transportation of blood samples for drugs analysis.

Another advantage of using DBS is because, at times, different small laboratories collaborate and centralized different drugs for more specific and sensitive analysis at different locations. In addition, the small volumes of blood used in DBS are a good fit for multiple small volume collections from different patients at suitable locations.

This method could ease sample collection and also the absence of “post” collection analysis due to microbial action on samples in one out of eight laboratories, “lab 4” as seen on (Fig.7C). Moreover, the samples can easily be stored at room temperature until 30 days without affecting the stability of PEth. Despite the increasing popularity and advantages of DBS over the conventional EDTA whole blood sample, it has limited sensitivity due to the small sample volume.
It could be noted here that, during the early days of the development of the two PEth molecular species recommended by [21] for clinical routine evaluations, only PEth-16:0/18:0 commercial reference substance IS, was assessable. In future, when PEth-16:0/18:2, reference material will be commercially available, it will therefore open a new field of application of PEth to uncover other undocumented variables such as dietary intake.

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