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Application of dispersive liquid–liquid microextraction for the determination of phosphatidylethanol in blood by liquid chromatography tandem mass spectrometry

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ABSTRACT

Phosphatidylethanol (PEth) is a phospholipid which requires for its metabolic formation the presence of relatively high ethanol levels. PEth is thus a promising marker to quantify ethanol abuse. Dispersive liquid–liquid microextraction has become a popular technique because it is fast, inexpensive, easy to operate and consumes low volume of organic solvent. In this method, the appropriate mixture of extraction solvent (230 μL dichloromethane) and disperser solvent (630 μL acetone) are injected into the sample by syringe, rapidly. The liquid chromatography method using a reversed phase-C8 column and a negative ion mode electrospray ionization tandem mass spectrometry detection instrument was developed for the determination of small amounts of PEth that might be present in blood samples, using phosphatidylbutanol (PBut) as an internal standard. The sensitivity of detection obtained with tandem MS was better than that of previous methods. Good linearity was obtained for a range of LOQ-10 μg/mL for PEth, whereas all of the deviations in precision and accuracy were less than 15% except for the LLOQ, where it should not exceed 20%. A set of 50 blood samples were analyzed by such method and whole blood concentrations of PEth 16:0/18:1 ranged from LLOQ to 1.71 μg/mL.

1. Introduction

Heavy drinking constitutes a significant problem in our societies as alcohol is the most popular legal drug used in our times [1,2]. Its chronic consumption is a known cause of many medical conditions due to the toxic effects of ethanol or its metabolism [3]. In addition, chronic heavy drinking during pregnancy often leads to the appearance of the fetal alcohol syndrome (FAS), the most well-known consequence of FASD (Fetal alcohol spectrum disorders). It is characterized by facial dysmorphology, reduced growth, neurological deficit, mental disorders, development disabilities and mental retardation [4,5]. Because of these reasons, the availability of reliable biological markers of ethanol consumption becomes an extremely important issue in both clinical and forensic settings to prove recent use of ethanol and/or chronic heavy drinking. It includes markers of alcohol related toxic effects, such as mean corpuscular volume (MCV) and gamma-glutamyltransferase (gGT); indirect markers, such as carbohydrate-deficient transferring (CDT) and 5-hydroxytryptophol (5-HTOL); and direct markers, including blood ethanol itself, as well as alcohol derivatives such as fatty acid ethyl esters (FAEE), ethyl glucuronide (EtG), ethyl sulfate (EtS), and phosphatidylethanol (PEth) [6].

The blood biomarker PEth is a phopholipid formed from phosphatidylcholine (PC) in cell membranes by a transphosphatidylation reaction catalyzed by phospholipase D (PLD) only in the presence of ethanol. Phospholipase D normally hydrolyzes PC into phosphatidic acid (PA) and choline, but because the affinity for ethanol is 1000 fold higher than for water, PEth is formed at the expense of PA when ethanol is present. The reaction is shown in Fig. 1 [2,7]. From the standpoint of the chemical structure, PEth is not a single molecule but a group of phospholipids with a common polar phosphoethanol head group onto which two fatty acid moieties are attached at positions sn-1 and sn-2. There exist many combinations of chain lengths with different numbers of double bonds leading as a result a large number of PEth molecular species; typically with a chain lengths of 16, 18 or 20 carbons. Phosphatidylethanol 16:0/18:1 (nomenclature for fatty acids =:[number of double bonds]) and 16:0/18:2 are the major fatty acid combinations in PC extracted from human erythrocyte membranes [8]. Recently published literature has shown the several molecular species of PEth

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exist in blood samples drawn from alcohol-dependent subjects [6]. It is measurable up to 15–20 days after last intake of alcohol in heavy drinkers according to Joya et al. [2]. The mean half life is about 4 days due to its slow degradation rate [1]. For all these reasons, it has been suggested that PEth could potentially be used as a marker of ethanol use [4,9]. Nevertheless, special care must be taken because the PLD enzyme is active at temperatures as low as −20 °C employed for storage purposes and consequently, PEth may be generated post-sampling if ethanol is present, potentially leading to false indications of prior heavy drinking [6]. The chemical structures of PEth and PBut and its possible structures on the monitored fragments are shown in Fig. 2.

Over the past few decades, different analytical techniques have been used to detect and quantify total PEth in biological matrices: thin layer chromatography (TLC), capillary electrophoresis, non-aqueous capillary electrophoresis (NACE) coupled to UV-detection, liquid chromatography (LC) coupled with an evaporative light-scattering detector (ELSD) or, more recently, LC electrospray ionization (ESI) mass spectrometry or multiple mass spectrometry (LC-MS and LC-MS/MS) [6].

The novelty of the method consists of using dispersive liquid–liquid microextraction (DLLME), introduced by Assadi and co-workers in 2006 [10], as the extraction method. It is a simple and fast microextraction technique based on the use of an appropriate extractant (an organic solvent with high density) and a disperser solvent with high miscibility in both extractant and aqueous phases. DLLME consists of two steps: (1) Injection of an appropriate mixture of extracting and disperser solvents into aqueous sample, containing the analytes. In this step, the extracting solvent is dispersed into the aqueous sample as very fine droplets and the analytes are enriched into it. After the formation of cloudy solution, the surface area between the extracting solvent and the aqueous sample becomes very large, so the equilibrium state is quickly attained and, therefore, the extraction time is very short. In fact, this is the principal advantage of this method. (2) Centrifugation of cloudy solution. After that, a sedimented phase is settled in the bottom of a conical tube. Other advantages include simplicity of operation, rapidity, low cost, high recovery, high enrichment factor and environmental benignity [11].

The aim of this study was to develop a liquid chromatography mass spectrometry method for the determination and quantification of PEth in blood using dispersive liquid–liquid microextraction (DLLME) as a new extraction method. The method was then applied to blood samples collected from heavy and social drinkers and makes it practical for routine laboratory use.

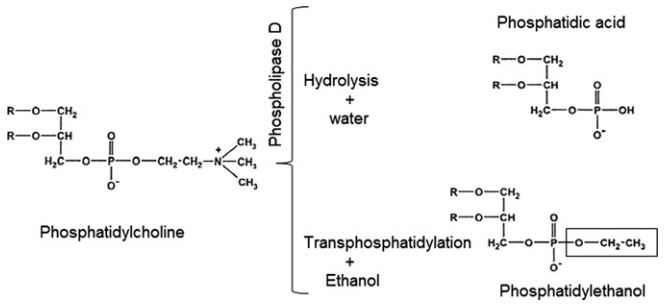

Fig. 1. Schematic figure of the formation of PEth via transphosphatidylation catalyzed by phospholipase D.

2. Material and methods

2.1. Chemicals

Phosphatidylethanol (16:0/18:1; 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanol; 98% purity; 10 μg/mL) and Phosphatidylbutanol (1-O-palmitoyl-2-O-oleoyl-sn-3-phosphobutanol; 98% purity; 10 μg/mL), used as internal standard, were purchased from Enzo Life Sciences (ELS) AG (Switzerland). Stock solutions were prepared in chloroform. Working solutions were prepared by dilution in mobile phase. All other chemicals were of HPLC-grade from Merck (Barcelona, Spain). Deionized water was obtained using a Millipore system Direct-Q3.

2.2. Blood samples

The blood specimens used for method development were obtained from clinical samples sent to our department for testing alcohol by GC-FID. Blank specimens used to develop and validate

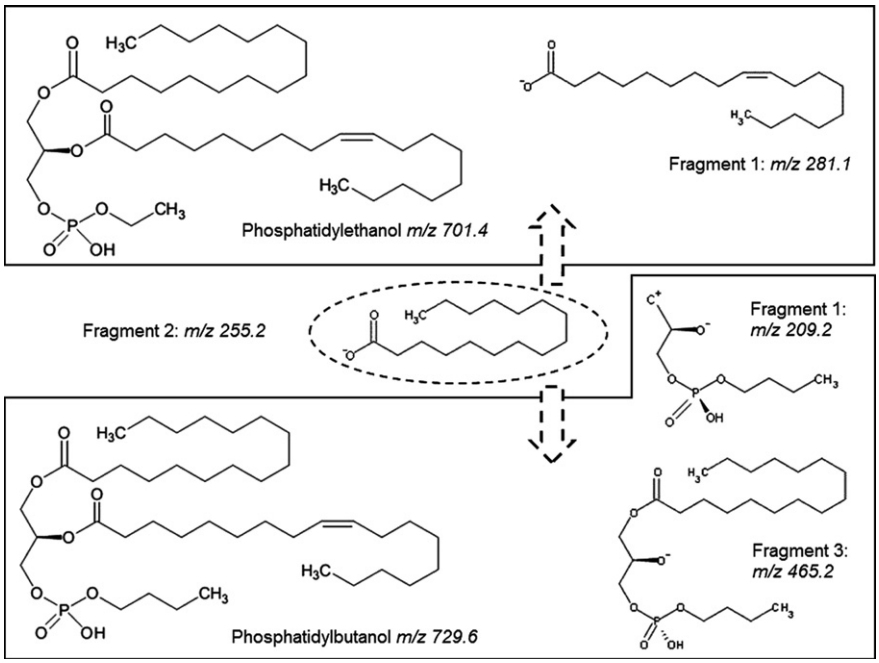

Fig. 2. Chemical structures of PEth and PBut.

the method were obtained from clinical samples but they were previously analyzed by GC-FID. A pool of negative blood analyzed by GC-FID was done and it was previously analyzed by this new method to confirm the negative results. The samples were collected in EDTA tubes and stored at 4 °C before analysis.

2.3. Sample preparation

Aliquots of 0.2 mL whole blood were used for analysis, spiked with PBut (50 μL Sol. 2 μg/mL). Methanol (950 μL) was added and the precipitation was removed by centrifugation (10 min). Then, it was diluted with water (1.4 mL) and a dispersive liquid–liquid microextraction (DLLME) was the following step. After an experimental design using StatGraphics, the best conditions were as follows: 630 μL of acetone (as disperser solvent) containing 230 μL of dichloromethane (as extracting solvent) was rapidly injected into sample and the mixture was gently shaken. Then, the mixture was centrifuged and the droplet formed was collected by a 100 μL syringe and transferred to a 8 mL glass vial. The organic solvent was evaporated under a stream of nitrogen in a heated aluminum block at 40 °C. The dried residue was redissolved with 100 μL of mobile phase (90% A-10% B) prior to injection of a 20 μL aliquot into the LC-MS/MS system.

2.4. Instrumentation

The LC tandem-MS system was an Agilent 1200 series (Agilent; Madrid; Spain) connected to Sciex API 4000 with the ESI interface operated in negative ion mode and Analyst 1.4.2 software (Applied Biosystems; Madrid, Spain). Data were adquired in the multiple reaction monitoring (MRM) mode: PEth-16:0/18:1 (m/z 701.4–255.2 and 281.1) and PBut (729.6–209.2, 255.3 and 465.2) (Table 1). The transitions marked with asterisk were used for quantification. For all the scans, the following conditions were set: collision gas 10 psi, curtain gas 10 psi, nebulizer gas 30 psi, auxiliary gas 60 psi, ion spray voltage −4500 V, temperature 450 °C, declustering potential −100 V, entrance potential −10 V, collision energy −50 V and collision cell exit potential −6 V (for list of parameters, see Table 1). Separation was performed by a Zorbax Eclipse XDB-C8 (2.1 × 50 mm; 3.5 μm; Agilent) maintained at 30 °C. LC system was operated in gradient mode with solvent A being 20% 2 mM ammonium acetate pH 7 and 80% acetonitrile and solvent B 100% isopropanol. From sample injection until 1 min, isocratic elution with 90% A and 10% B was used; from 1–3 min, a linear gradient to 50% A; from 3–5 min, a linear gradient to 100% B; from 5–6.5 min, a linear gradient back to 90% A and from 6.5–8.5 min, 90% A. The total flow rate was set to 0.3 mL/min and the sample injection volume, 20 μL.

3. Results

3.1. Validation procedure

Validation was achieved according to the FDA Guideline for bioanalytical method validation [12]. The suitability of the method for quantitative analysis was studied by testing selectivity, linearity and sensitivity, precision and accuracy, process efficiency, matrix effect and recovery.

3.1.1. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products and medication and other exogenous xenobiotics [12].

The selectivity was estimated by analyzing blood samples ($n=6$) collected from different teetotalers. No interfering peaks were found at the retention time for PEth and PBut.

3.1.2. Linearity

The calibration curve was created using a blank sample and spiked samples with PEth at concentration of 0.03, 0.5, 1, 2, 5, 7 and 10 μg/mL. All standard samples were spiked with internal standard (2 μg/mL). The curve was obtained by fitting the ratio of the peak areas of PEth 16:0/18:1 to that of IS versus concentrations. A linear response was observed in the range 0–10 μg/mL with a good correlation coefficient (0.998).

The LOD, defined as the lowest concentration giving a response of at least three times relationship S/N, was 0.01 μg/mL. The lowest standard on the calibration curve, LLOQ, should be accepted as the limit of quantification if the analyte response is at least 10 times the response compared to blank response. Moreover, analyte peak should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80–120% [12]. It was 0.03 μg/mL.

3.1.3. Precision and accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. It is determined by replicate analysis

Table 2
Intra and inter day-assay.

Spiked amount of PEth	0.03 μg/mL		0.5 μg/mL		5 μg/mL	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
CV	16.12	18.21	10.95	14.45	11.89	8.56
Relative error	3.82	5.43	8.53	6.27	0.02	2.42

Intra and inter day-assay.

Table 1
Mass spectrometric parameters for the LC-ESI-MS/MS method for quantitative and qualitative measurement of PEth in blood. Analysis was performed using ESI in negative ion mode $[M-H]^-$. List of the MRM parameters.

	Precursor ion, [m/z]	Daughter ion, [m/z]	Declustering potencial [V]	Collision energy [eV]	Entrance potential [V]	Collision cell exit potential [V]	Retention time (min)
PEth 16:0/18:1	701.4	255.2	−100	−50	−10	−6	2.5
		281.1*	−100	−40	−15	−6	
PBut 16:0/18:1	729.6	209.2	−100	−50	−10	−3	3.1
		255.3*	−100	−50	−15	−10	
		465.2	−90	−40	−10	−10	

Mass spectrometric parameters for the LC-ESI-MS/MS method for quantitative and qualitative measurement of PEth in blood. Analysis was performed using ESI in negative ion mode $[M-H]^-$. List of the MRM parameters.

of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is

Table 3
Matrix effect (ME), Recovery (RE) and Process Efficiency (PE) data for PEth (1) and PBut (2).

	Mean peak area											
	1			2			ME [%]		RE [%]		PE [%]	
Concentration [μg/mL]	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	1	2	1	2	1	2
0.5	4,44E+07	1,34E+07	6,22E+06	2,10E+07	4,85E+06	3,60E+06	30,22	23,09	66,38	74,16	14,02	17,13
5	1,44E+08	1,13E+08	4,92E+07	1,74E+07	1,01E+07	3,54E+06	78,42	57,67	73,54	75,18	34,14	20,29

Matrix effect (ME), Recovery (RE) and Process Efficiency (PE) data for PEth (1) and PBut (2).

Table 4
Subjects characteristics ($n=50$).

Subjects	Gender	Age	Alcohol intake	PEth [μg/mL]	Ethanol concentration GC-FID [g/L]
1	M	21	–	0.08	0.69 (B); 1.16 (U)
2	M	47	Alcoholic	1.71	3.64 (B); 4.38 (U)
3	M	42	–	1.12	4.34 (B); 6.43 (U)
4	M	53	Alcoholic	Traces	3.00 (B); 2.42 (U)
5	F	38	Alcoholic	1.1	4.57 (B); 4.57 (U)
6	M	50	–	0.28	2.32 (B); 2.58 (U)
7	M	57	Possible alcohol dependence	0.82	1.60 (B); 1.84 (VH)
8	M	26	–	0.63	1.08 (B); 1.46 (U)
9	M	76	–	0.9	2.75 (B); 2.94 (VH)
10	M	61	–	0.43	2.18 (B); 2.78 (U)
11	M	66	Possible alcohol dependence	0.13	2.88 (B); 3.25 (VH)
12	M	52	–	0.68	3.21 (B); 4.20 (U); 3.78 (VH)
13	M	61	–	0.36	3.27 (B); 3.33 (VH)
14	M	66	–	0.39	1.12 (B); 1.33 (U); 1.22 (VH)
15	M	51	Teetotalers	0	NEG
16	M	74	Alcoholic	0.06	NEG
17	F	47	Teetotalers	Traces	NEG
18	M	56	Alcoholic	0.42	NEG
19	M	44	–	0.51	3.8 (B); 4.33 (VH)
20	F	51	Teetotalers	0	NEG
21	M	53	–	0.16	1.81 (B); 2.31 (U)
22	M	58	–	0.09	NEG
23	M	70	Teetotalers	0	NEG
24	M	66	–	0.08	NEG
25	M	35	–	0.03	NEG
26	F	53	–	0.06	1.28 (B)
27	M	82	–	0	NEG
28	F	82	–	0	NEG
29	M	58	–	Traces	NEG
30	F	61	Possible alcohol dependence	0.07	NEG
31	F	75	–	Traces	NEG
32	M	50	–	0	0.18 (B)
33	F	62	–	Traces	NEG
34	F	69	–	Traces	NEG
35	M	57	–	Traces	0.14 (B); 0.32 (U)
36	M	64	–	Traces	0.79 (B); 0.86 (U)
37	F	54	–	Traces	NEG
38	M	47	–	0.23	NEG
39	F	72	–	0	NEG
40	M	53	–	Traces	NEG
41	M	54	–	0.05	NEG
42	M	61	–	Traces	0.19 (B)
43	M	55	–	0.05	2.9 (B); 2.95 (U); 3.1 (VH)
44	M	67	–	Traces	NEG
45	M	27	–	0.05	0.63 (B)
46	M	76	–	0	NEG
47	M	61	–	0.05	NEG
48	M	49	–	0.06	1.86 (B); 2.04 (VH)
49	M	60	–	0.81	2.86 (B); 2.91 (VH)
50	M	–	–	Traces	NEG

Subjects characteristics ($n=50$; B: blood; U: urine; VH: vitreous humor).

recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV [12].

Intra-day and inter-day assay was evaluated by performing repeated injections (n=5) of the same sample at three concentrations (0.03, 0.5 and 10 μg/mL) in the same day and on five different days respectively. Data presented in Table 2 satisfy the international validation rules. The CV and relative error are less than 15% (14.45 and 8.53% are the highest values, respectively), except for at the LLOQ where they are within the accepted 20% of deviation (18.21 and 5.43% are the highest values for CV and relative error, respectively).

3.1.4. Process efficiency, matrix effect and recovery

Three sets of two concentrations and repeated three times at each concentrations (it is represented its average in the table) were prepared to evaluate the recovery, process efficiency and the absence or presence of matrix effect as described Matuszewski et al. [13]. The first set was prepared to evaluate the MS/MS response for neat standards of two analystes (PEth and PBut) injected in the mobile phase. The second set (set 2) was prepared in blood extracts spiked after extractions. The third set (set 3) was prepared in blood samples but spiked before extraction. The results of the analyses for sets 1–3 are summarized in Table 3.

The results obtained in this manner allow determination of the matrix effect (ME), recovery (RE) of the extraction procedure and process efficiency (PE) by comparing the absolute peak areas for PEth and PBut obtained in sets 1–3 (Table 3). Peak areas obtained in neat solutions standards in set 1 are depicted as A, the corresponding peak areas for standards spiked after extraction into blood samples as B (set 2), and peak areas for standards spiked before extraction as C (set 3). The ME, RE and PE values can be calculated according to Eq. (1)–(3) [13].

$$ME(\%) = B/A \times 100 \quad (1)$$

$$RE(\%) = C/B \times 100 \quad (2)$$

$$PE(\%) = C/A \times 100 = (ME \times RE) \times 100 \quad (3)$$

3.2. Application to real samples

The capabilities of the proposed method of identifying and quantifying PEth, a marker of alcohol, was tested on blood samples collected from clinical samples. Some information is described in Table 4. It can be seen that fifty blood samples were applied to the method and all of them were previously analyzed by GC-FID for the determination of ethyl alcohol. Following this technique, we found samples 1–14; 19; 21; 26; 36; 43; 45; 48 and 49 with positive results. It agrees with our results except for samples 4 and 36 that we found them as negative. Information about alcohol consumption is not easy to obtain, but we know that samples 2, 4, 5, 16 and 18 belong to alcoholic people, as can be supported by our results. Positive results obtained by HPLC-MS/MS ranges between 0.046 to 1.706 μg/mL (higher than 0.03 μg/mL; a value established as cut-off). It is shown a bar chart in Fig. 3 from these values. A chromatogram of real case number 7 is showed below (Fig. 4).

4. Discussion

Biological markers of ethanol consumption are widely used and are needed in a number of situations where alcohol abuse may occur and should be avoided. Detection in medical practises, during treatment of recovering alcoholics, during pregnancy and also for forensic purposes are examples of such situations. PEth has a mean half-life of about four days in blood of alcoholics, and it was still measurable after up to 2–3 weeks of sobriety. Because of its high specificity and slow elimination it has been proposed as a marker for alcohol abuse [2,14–17].

Traditionally, the most used analytical method was HPLC-ELSD but HPLC-MS or tandem spectrometry were used since 2009. This caused lower LOD and LOQ and better recoveries; for instance, 33% from Gnann et al. [18] versus Nalesso or Faller et al. [6,19]. It is a common practise to determine the recovery of a compound extracted from a matrix by comparing the response of a compound spiked into a biological sample, extracted, reconstituted in a solvent and injected with the corresponding peak areas of the same compound injected directly in the same solvent. It would be equivalent to the recovery

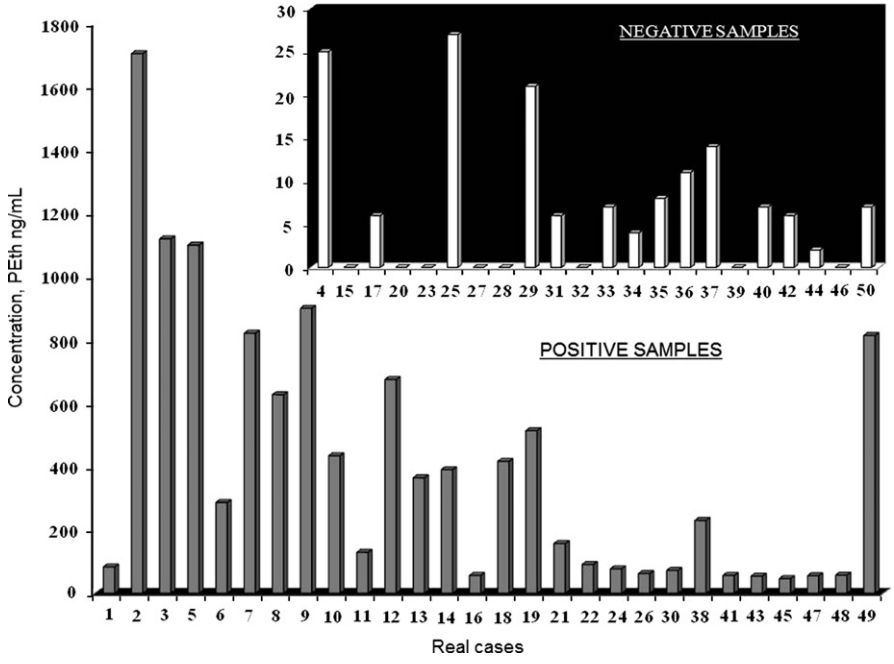

Fig. 3. Bar chart from real cases.

being determined as Eq. (3). However, this practise may not be correct since it does not take into account the matrix effect that may greatly influence this ratio. Eq. 3 (PE) may be instead considered as the overall process efficiency [13]. LOD and LOQ were improved highly; from 1998 until 2009 we can found these values much higher than nowadays. At present, it ranges between 0.017 and 0.054 μg/mL for LOD and LOQ respectively except for those values found by Nalesso et al. (0.0005 and 0.001 μM) [6], which were quite different from the others values. Range of concentrations show differences in all these reviewed years. In this case, the higher point chosen for the calibration curve was 10 μg/mL as it is in accordance with values showed in earlier reports. All these data can be seen in Table 5 [20–30].

The mean absolute matrix effect, calculated according to Eq. (1) was 30.22 and 23.09 (for a concentration of 0.5 μg/mL) and 78.42

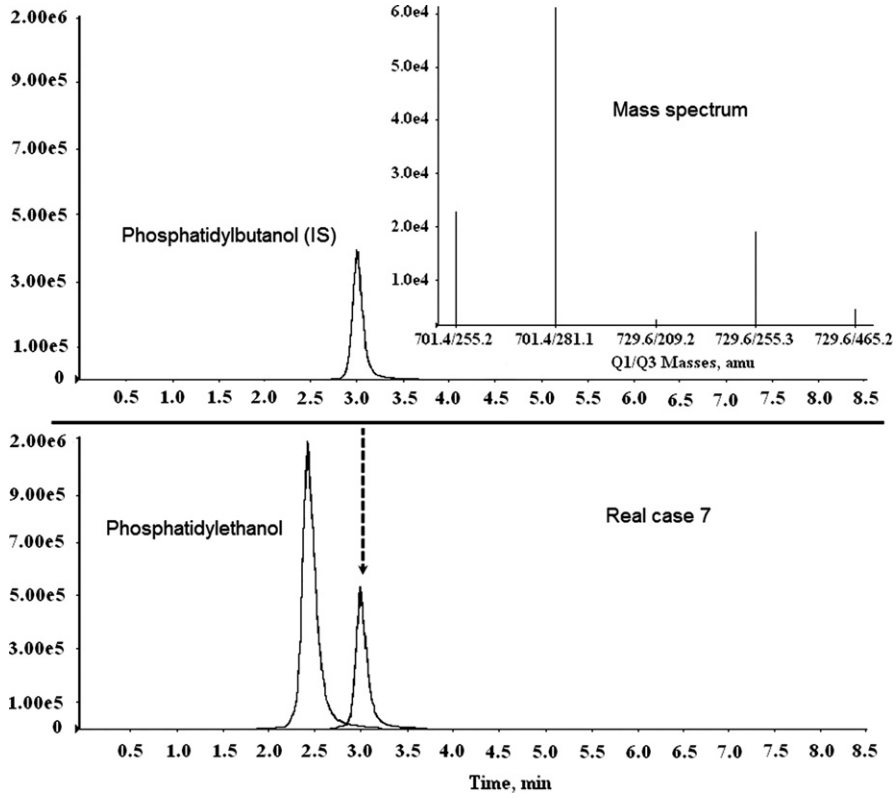

Fig. 4. Chromatogram of a blank sample and a real case.

Table 5
Review of articles using blood as biological matrix for PEth.

Ref.	Blood (mL)	Extraction technique	Analytical method	LOD [μM]	LOQ [μM]	Range of concentrations [μM]	Recovery (%)	Real cases [μM]
Nalesso A. 2011	0.3	LLE	LC-MS/MS	0.0005	0.001	0.001–2.0	80	–
Faller A. 2011	0.1	LLE	LC-MS/MS	0.02*	0.08*	0.05–5*	68.9 90.7	0.92–213*
Zheng Y. 2011	0.1	LLE	LC-MS/MS	0.009	0.1	0.05–2.48	–	LOD-18.7
Stewart S. 2010	1	LLE	LC-MS/MS	–	0.02*	–	–	–
Gnann H. 2010	0.3	LLE	LC-MS/MS	0.04	–	–	–	–
Nalesso A. 2010	0.3	LLE	NACE-ESI-MS	0.1	0.4	0.4–10	–	–
Stewart S. 2009	10	–	LC-MS/MS	–	0.02*	–	–	–
Helander A. 2009	0.1	LLE	LC-MS	< 0.02	< 0.1	0.2–20.0	–	0.1–21.7
Gnann H. 2009	0.3	LLE	LC-MS/MS	0.02*	0.05*	0.02–2*	33	–
Comasco E. 2009	–	–	–	–	0.25	–	–	–
Marques P. 2009	–	–	–	–	0.22	–	–	–
Kip M.J. 2008	–	–	HPLC-ELSD	–	0.22	–	–	–
Bendroth P. 2008	–	–	HPLC-ELSD	–	0.22	–	–	0.33–19
Varga A. 2008	0.3	LLE	NACE-UV	0.4	–	0–10	–	–
Aradottir S. 2006	0.3	LLE	HPLC-ELSD	–	0.22	0–16	–	–
Aradottir S. 2005	0.3	LLE	HPLC-ELSD	–	0.2	0.2–4	–	0.5–12
Aradottir S. 2004	1–0.3	LLE	HPLC-ELSD	–	–	–	–	–
Varga A. 2002		LLE	HPLC-ELSD	0.2	–	–	–	-
Hansson P. 2001	0.3	LLE	HPLC-ELSD	0.8	–	–	–	0.8–22
Varga A. 2000	0.3	LLE	HPLC-ELSD	–	–	–	–	–
Yon C. 2000	–	–	GC–MS	–	–	–	–	–
Gunnarsson T. 1998	4	LLE	HPLC-ELSD LC-MS	0.2	–	–	–	–
This method	0.2	DLLME	LC-MS/MS	0.01*	0.03*	0–10*	70	LLOQ-0.002*

* μg/mL.

and 57.67 (for a concentration of 5 μg/mL) for PEth and PBut, respectively. A value of 100% indicates that the response in the mobile phase and in the blood extracts were the same and no absolute matrix effect was observed. A value of > 100% indicates an ionization enhancement and a value of < 100%, as this case, indicates an ionization suppression. The presence of an absolute or even a relative matrix effect for a given analyte (PEth in this case) does not necessarily indicate that the bioanalytical method may not be valid. Assuming the relative matrix effect exhibits the same pattern for the drug and the internal standard in all lots studied, the drug-to-internal standard ratio, a measure of the drug concentration, should not be affected.

The sample preparation step in an analytical process typically consists of an extraction procedure that results in the isolation and enrichment of components of interest from a sample matrix. Regarding this, liquid–liquid extraction (LLE) is the only extraction used by all authors. LLE is among the oldest of the preconcentration and matrix isolation techniques in analytical chemistry. However, LLE is time-consuming and requires large amounts of organic solvent. Solid phase extraction (SPE) uses much less solvent than LLE, but can be relatively expensive. Supercritical fluid extraction (SFE) can also be relatively expensive. Compared with LLE, SPME is a solvent free process that includes simultaneous extraction and preconcentration of analytes from aqueous samples or the headspace of the samples. However, SPME is also expensive, its fiber is fragile and has limited lifetime and sample carry over can be a problem. We improved this analytical procedure by using DLLME. It was found to be an alternative extraction method to LLE with good recoveries (around 70%). DLLME was demonstrated as a novel microextraction technique with a high performance and powerful preconcentration method. In this method, the appropriate mixture of extraction solvent and disperser solvent is injected into aqueous sample rapidly. Thereby, cloudy solution is formed. In fact, it is consisted of fine particles of extraction solvent which is dispersed entirely into aqueous phase. After centrifugation, the fine particles of extraction solvent are sedimented in the bottom of the conical test tube. Some important parameters, such as kind of extraction and disperser solvent and volume of them, and extraction time were investigated. The advantages of DLLME method are simplicity of operation, rapidity, low cost, high recovery and enrichment factor [10].

5. Conclusions

In the present study, a new extraction technique (DLLME) was described. It is fast, simple and inexpensive method. Good recoveries within a short time were got. The LC-MS/MS method with C8 column was developed for analyzing very small amounts of PEth. The results show that this technique is highly sensitive to determine PEth at low concentrations. The samples used here were human blood from clinical samples. The total analysis time was 8.5 min. Lower detection limits were obtained compared to published articles. The method provides high precision and accuracy within the linear range of detection. Data presented in this article suggest that PEth may be considered as an excellent candidate for a sensitive and specific marker of alcohol intake.

References

[1] H. Gnann, C. Engelmann, G. Skopp, M. Winkler, V. Auwärter, S. Dresen, N. Ferreirós, F.M. Wurst, W. Weinmann, Anal. Bioanal. Chem. 396 (7) (2010) 2415–2423.
[2] X. Joya, B. Friguls, S. Ortigosa, E. Papaseit, S.E. Martínez, A. Manich, O. García-Algar, R. Pacific, O. Vall, S. Pichini, J. Pharm. Biomed. Anal. 69 (0) (2012) 209–222.
[3] S.H. Stewart, A. Reuben, A. Brzezinski, D. Koch, J. Basile, P. Randall, P. Miller, Alcohol Alcohol. 44 (5) (2009) 464–467.
[4] S.H. Stewart, T.L. Law, P.K. Randall, R. Newman, Alcohol.—Clin. Exp. Res. 34 (3) (2010) 488–492.
[5] C. Lupton, L. Burd, R. Harwood, Am. J. Med. Genet. C. (Semin. Med. Genet.) 15 (1) (2004) 42–50.
[6] A. Nalesso, G. Viel, G. Cecchetto, D. Mioni, G. Pessa, D. Favretto, S.D. Ferrara, J. Chromatogr. A. 1218 (46) (2011) 8423–8431.
[7] E. Comasco, N. Nordquist, J. Leppert, L. Oreland, R. Kronstrand, C. Alling, K. Nilsson, J. Stud. Alcohol Drugs 70 (5) (2009) 797–804.
[8] A. Helander, Y. Zheng, Clin. Chem. 55 (7) (2009) 1395–1405.
[9] S. Aradottir, B. Olsson, BMC Biochem. 6 (1) (2005) 18.
[10] M. Rezaee, Y. Assadi, M. Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, J. Chromatogr. A 1116 (2006) 1–9.
[11] M. Rezaee, Y. Yamini, M. Faraji, J. Chromatogr. A. 1217 (16) (2010) 2342–2357.
[12] Food and Drug Administration, U.S. Department of Health and Human Services, Guidance for Industry, Bioanalytical Method Validation, FDA. ⟨http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf⟩, 2011 (accessed 29.10.12).
[13] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (13) (2003) 3019–3030.
[14] F.M. Wurst, B. Tabakoff, C. Alling, S. Aradottir, G. Wiesbeck, F. Müller-Spahn, F. Pragst, B. Johnson, M. Javors, N. Ait-Daoud, G. Skipper, C. Spies, Y. Nachbar, O. Lesch, K. Ramskogler, S. Hartmann, M. Wolfersdorf, Alcoholism-Clin. Exp. Res. 29 (7) (2005) 1268–1275.
[15] P. Hansson, A. Varga, C. Krantz, Int. J. Leg. Med. 115 (3) (2001) 158–161.
[16] F. Pragst, K. Spiegel, F. Sporkert, M. Bohnenkamp, Forensic Sci Int. 107 (1-3) (2000) 201–223.
[17] T. Gunnarsson, A. Karlsson, P. Hansson, G. Johnson, C. Alling, G. Odham, J Chromatogr B. 705 (2) (1998) 243–249.
[18] H. Gnann, W. Weinmann, C. Engelmann, F.M. Wurst, G. Skopp, M. Winkler, A. Thierauf, V. Auwärter, S. Dresen, N.F. Bouzas, J. Mass Spectrom. 44 (9) (2009) 1293–1299.
[19] A. Faller, B. Richter, M. Kluge, P. Koenig, H. Seitz, A. Thierauf, H. Gnann, M. Winkler, R. Mattern, G. Skopp, Anal. Bioanal. Chem. 401 (4) (2011) 1163–1166.
[20] Y. Zheng, O. Beck, A. Helander, Clin. Chim. Acta 412 (2011) 1428–1435.
[21] A. Nalesso, G. Viel, G. Cecchetto, G. Frison, S.D. Ferrara, Electrophoresis 31 (2010) 1227–1233.
[22] P. Marques, S. Tippetts, J. Allen, M. Javors, C. Alling, M. Yegles, F. Pragst, F. Wurst, Addiction 105 (2009) 226–239.
[23] M.J. Kip, C.D. Spies, T. Neumann, Y. Nachbar, C. Alling, S. Aradottir, W. Weinmann, F.M. Wurst, Alcohol Clin. Exp. Res. 32 (7) (2008) 1284–1291.
[24] P. Bendroth, R. Kronstrand, A. Helander, J. Greby, N. Stephanson, P. Krantz, Forensic Sci. Int. 176 (2008) 76–81.
[25] A. Varga, Staffan Nilsson, Electrophoresis 29 (2008) 1667–1671.
[26] S. Aradottir, G. Asanovska, S. Gjerss, P. Hansson, C. Alling, Alcohol. Alcohol. 41 (4) (2006) 431–437.
[27] S. Aradottir, K. Moller, C. Alling, Alcohol. Alcohol. 39 (1) (2004) 8–13.
[28] A. Varga, C. Alling, J. Lab. Clin. Med. 140 (2002) 79–83.
[29] A. Varga, P. Hansson, G. Johnson, C. Alling, Clin. Chim. Acta 299 (2000) 141–150.
[30] C. Yon, J.S. Han, Exp. Mol. Med. 32 (4) (2000) 243–245.