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CASE REPORT

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Phosphatidylethanol in post-mortem blood as a marker of previous heavy drinking

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Abstract Phosphatidylethanol (PEth) is an ethanol-phospholipid adduct, formed via non-oxidative metabolism of ethanol. PEth was measured in femoral blood from 85 consecutive forensic autopsies and was detected in 35 of the cases at concentrations ranging from 0.8 to 22.0 μmol/l. Of the PEth positive cases, 12 did not have significant levels of ethanol in the blood. Two cases (both suicides involving hanging) had detectable ethanol, but no PEth present in the blood. We conclude that measurements of PEth provide indications of previous alcohol abuse in cases where this may not otherwise be evident.

Keywords Phosphatidylethanol · Alcoholism · Diagnosis · Forensic · Autopsy

Introduction

Alcohol plays a highly significant role in forensic medicine as a direct or indirect cause of death and the quantification of ethanol is the most common forensic biochemical test [1]. However, its interpretation is not always straightforward. One confounding factor is the rapid clearance of alcohol, especially in high consumers [2]. One can therefore expect a significant number of alcohol-related deaths not to display alcohol in blood [3] so that tests are needed to assess previous alcohol abuse.

Phosphatidylethanol (PEth, Fig. 1) is a "pathological" phospholipid, formed only in the presence of ethanol via the action of phospholipase D, formed at the expense of the natural product phosphatidic acid [4, 5, 6, 7, 8]. PEth has been demonstrated in the brain tissue of ethanol-exposed rodents [9]. In human subjects, PEth was found in blood of manifest alcoholics up to 2 weeks after admission to a confined clinic, whereas blood ethanol was zero even on the first day after admission [10]. Blood PEth is found almost exclusively in the erythrocyte fraction [11]. A single dose of alcohol (even one producing significant inebriation) is not enough to make PEth detectable in blood [12]; extrapolating from data obtained from healthy students, it seems that a daily consumption of at least 50 g of ethanol for several days are necessary for the blood PEth to reach a measurable level [12]. A theoretical disadvantage is the possibility of PEth formation post-mortem, since phospholipase D is active even at freezer temperatures [13].

This study was performed to evaluate PEth assays in forensic medicine as a possible marker of previous alcohol abuse.

Materials and methods

Autopsies and blood sampling

We studied 85 consecutive autopsies carried out at the Department of Forensic Medicine during two periods in 1998 and 1999. We excluded homicide cases, severely decomposed bodies, cases where blood ethanol was not measured and cases with significant blood loss, where blood could not easily be obtained. Blood was obtained from puncture of the femoral vein and stored refrigerated in plastic tubes until further processed. The storage period was 4–16 h and separate control experiments ascertained that blood can be stored for up to 3 days at refrigeration temperatures without affecting levels of PEth (cf. Köhler et al. [14]). The above procedures were done in accordance with recommendations on ethics by the Swedish National board of forensic medicine.

Lipid extraction and PEth analysis

A 300 μl aliquot of blood was added under agitation to 10 ml of hexane:isopropanol (3 : 2 v/v) and further agitated briskly for about 5 s. Precipitated protein was removed by centrifugation at 1,500 *g* for 10 min, the extract was then dried under nitrogen and then re-

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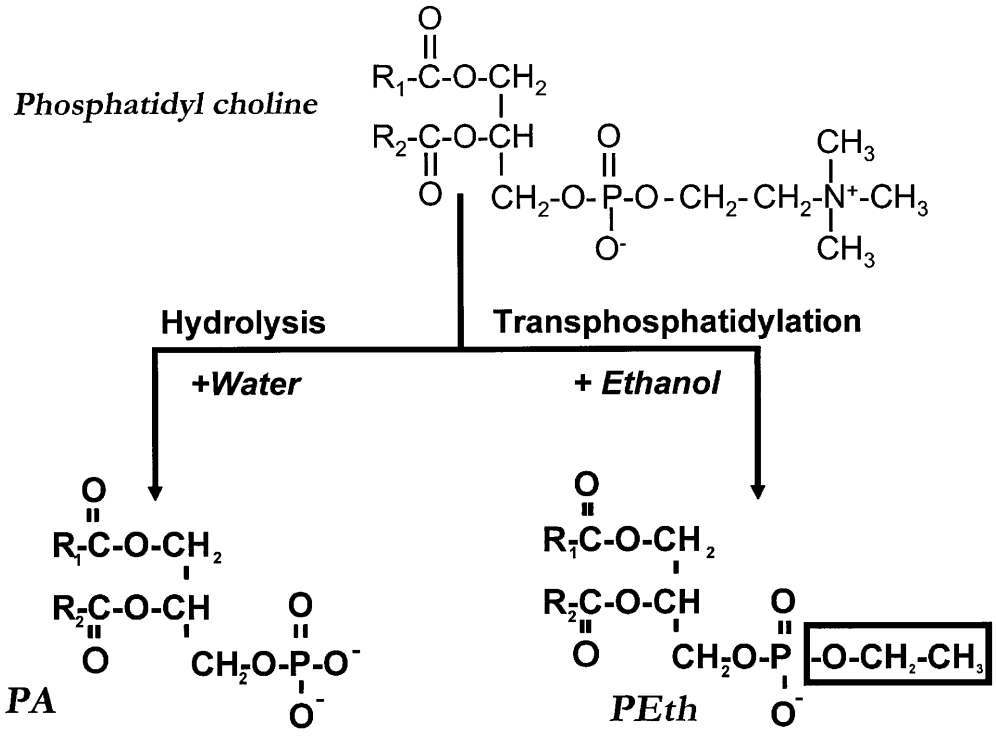

Fig 1 Schematic figure of the formation of phosphatidylethanol via transphosphatidylation catalysed by phospholipase D (*PA* phosphatidic acid, *PEth* phosphatidylethanol)

suspended in 150 µl of hexane:isopropanol. PEth was analysed by using high-performance liquid chromatography with an Alltech 500 evaporative light scattering detector. A tertiary gradient consisting of solutions A (hexane), B (1-propanol:water 85 : 15 v/v) and C (1-propanol : acetic acid : triethylamine 316 : 16 : 1 v/v/v), was used at a flow rate of 1.0 ml/min. The gradient was run according to the following scheme: 0–3 min: A 85%, B 1%, C 14%; 3–8 min: A 85–63%, B 1–23%, C 14%; 8–12 min: A 63–36%, B 23–50%, C 14%; 12–13 min: A 36–0%, B 50–86%, C 14%; 13–23 min: A 0%, B 86%, C 14%; 23–28 min: A 0–85%, B 86–1%, C 14%; 28–58 min: A 85%, B 1%, C 14%. The temperature in the detector was set at 80°C, and a gas flow of 2 l/min was used. The detector was calibrated with phosphatidylethanol, synthesised from egg yolk phosphatidylcholine and ethanol by plant phospholipase D [15]. Phosphatidylcholine and phospholipase D were purchased from Sigma-Aldrich (Sweden). Contents of phosphatidylethanol were quantified as lipid-bound phosphorus [16]. The detection limit for blood PEth was previously found to be 0.8 µmol/l [12].

Ethanol analysis

Blood ethanol was analysed by the National Swedish Forensic Chemistry Laboratory. The method used employs headspace gas chromatography on two different instruments, reporting the mean of the two results. Maximum allowable variation between results is 10% and the detection limit of the method is 0.1 mg/ml.

Liver histology

The presence of fatty liver and/or cirrhosis as an indirect marker of alcoholism was judged from histological slide specimens stained with hematoxylin-eosin and/or Scharlach Rot. This evaluation was done blindly in relation to the PEth data.

Data analysis

Data were compiled from autopsy identification numbers. Anamnestic evidence of alcoholism, drug abuse etc., as well as laboratory and histological findings were also obtained by autopsy identification number, from the database of the National Swedish Board of Forensic Medicine. This database includes police records, medical records and occasionally information from social authorities. Statistical analysis (means, standard deviations, correlation coefficient and Mann-Whitney unpaired test) was performed by the StatView 4.5 software on a Macintosh computer.

Table 1 Description of the autopsy material (*n.s.* no significant difference)

Group	Number *(n)*	Notes
Total material	85	Consecutive autopsies except exclusions defined in the text, 31 cases had anamnestic records of alcohol abuse
Detectable PEth	35	Blood PEth 7.9 ± 6.2 µmol/l (range 0.8–22.0)
Detectable ethanol	27	Blood ethanol 1.8 ± 1.2 mg/ml (range 0.2–4.1), includes one fatal methanol poisoning (blood methanol 0.36 mg/ml)
Detectable PEth and ethanol ≤ 0.2 mg/ml	12	See Table 2. Blood PEth 6.2 ± 6.5 µmol/l (range 1.1–22.0)
Detectable PEth and ethanol > 0.2 mg/ml	23	Blood PEth 8.9 ± 6.0 µmol/l (range 0.8–19.9; n.s. versus above group)
Detectable ethanol without detectable PEth	2	Two cases of suicide (hanging). Blood ethanol 0.7 and 1.1 mg/ml

Table 2 Cases with detectable phosphatidylethanol (PEth) in blood, without significant levels of ethanol (= 0.2 mg/ml, the legal driving limit in Sweden, *M* male, *F* female)

Case number; sex, age (years)	Blood PEth (μmol/l)	Blood ethanol (mg/ml)	Short description
1. F, 71	1.1	0	Psychiatric disease, fatal sigmoiditis and peritonitis
2. F, 56	1.5	0	Psychiatric disease, overdose of analgesics and psychoactive drugs
3. M, 53	10.1	0	Anamnestic alcoholic, no certain cause of death, liver cirrhosis
4. M, 46	13.1	0	Anamnestic alcoholic and possibly drug addict, massive fatty liver (hepatitis?)
5. M, 74	1.7	0	Brain hemorrhage and fatal injuries after fall, moderately fatty liver
6. M, 38	22.0	0	Anamnestic alcoholic and epileptic
7. M, 59	8.4	0.2	Possible alcoholic, intoxication clomipramine and amitriptyline
8. M, 61	1.6	0	Anamnestic alcoholic, no certain cause of death, fatty liver
9. M, 59	1.2	0	Car accident, fatty liver
10. M, 40	1.1	0	Marijuana user, moderately fatty liver
11. M, 64	6.8	0.2	Anamnestic alcoholic, fatal myocardial infarction
12. M, 47	5.5	0	Anamnestic alcoholic, no certain cause of death, severe fatty liver and cirrhosis

Results

Of the 85 cases, 35 had detectable PEth in the femoral blood at autopsy (Table 1), ranging from just detectable (0.8 μmol/l) to very high (22.0 μmol/l). Out of these cases, 12 displayed absent or insignificant (≤ 0.2 mg/ml) levels of blood ethanol (Table 2). Mean PEth levels were slightly higher in those cases where ethanol was present, but there was no significant difference between the groups (Table 1) and there was no correlation between measurable concentrations of ethanol and PEth levels (data not shown).

One case of fatal methanol poisoning displayed a chromatographic peak at the position of PEth, representing phosphatidylmethanol, and it is known that phospholipase D also accepts other alcohols as co-substrates [17]. We cannot exclude the co-existence of phosphatidylmethanol and phosphatidylethanol within the peak. The case is included in the PEth-positive group.

Two cases, both suicides involving hanging, had detectable ethanol but no detectable PEth in blood (Table 1).

Discussion

This study shows that phosphatidylethanol (PEth) is measurable in post-mortem blood at forensic autopsy and is present in a significant proportion of the cases studied. PEth may represent an improvement in the diagnosis of alcoholism in cases where this may otherwise be difficult [3, 18, 19]. A potential interpretation problem is the possibility of PEth formation post-mortem [13]. However, at least in the 12 cases presented in Table 2, this possibility may be discarded, since ethanol was not present in blood to act as co-substrate for phospholipase D. We therefore conclude that in these cases, blood PEth was synthesised ante-mortem. The factors influencing PEth turnover are not well investigated. The rate of PEth formation might be influenced by previous ethanol experience, since rats repeatedly injected with ethanol formed more PEth in the brain at a given dose of ethanol, than did ethanol-naive rats [9]. The amounts and activity of phospholipase D isoenzymes vary among organs and so do the GTPases that regulate crucial enzymes in PEth formation [20]. The formation rate in human blood has not yet been studied, however, we found the half-life to vary between 3.0 and 5.5 days and there was also some evidence for a relationship between initial PEth concentrations and the half-life [11]. Further studies on PEth metabolism in normal controls and alcoholics are needed.

High PEth concentrations (> 5 μmol/l) were found in several of the cases (Table 2), in some cases higher than we found in known alcoholics during detoxication [10, 12]. We previously found that a person needs to consume a minimum of about 50 g of ethanol daily for several days, before PEth becomes detectable in the blood [12]. Thus, the patients with high levels of PEth are very likely to have been alcoholics. We can also confirm this hypothesis, since all of them had either police or other records of alcohol abuse.

In the quest for hidden alcohol abuse, it may be more interesting to scrutinise the cases where only small amounts of PEth were detectable. Cases nos. 5, 9 and 10 in Table 2 had no anamnestic record of abuse, but displayed low but clearly detectable levels of PEth. All three also displayed some degree of fatty liver by routine histology. We interpret these findings to reflect a minor degree of alcohol abuse, which might for instance accompany a period of depression.

Formation of PEth is one aspect of the non-oxidative metabolism of ethanol, which also results in production of other “pathological” metabolites. Some of these have been evaluated as potential marker molecules. Fatty acid ethyl esters [21, 22] are readily detectable in blood after alcohol intake, but display a short time-window for detection, 24 h or less and correct sample storage is also important to avoid false positive results [23]. Other investigators have instead used measurements on hair samples and this medium may be preferable to blood for analysis of fatty acid ethyl esters [24].

Ethyl glucuronide is another non-oxidative ethanol adduct which has attracted interest as a possible marker of abuse. It is found in urine up to 80 h after alcohol intake and the analysis appears to be sensitive enough to detect a single bout of drinking [25]. Ethyl glucuronide can also be detected in other tissues including hair [26, 27, 28]. In comparison to ethyl glucuronide, PEth only becomes detectable after more prolonged alcohol intake [12] but remains detectable for a longer time, especially in manifest alcoholics [11]. These two analyses may therefore complement one another diagnostically [29].

Carbohydrate-deficient transferrin (CDT) is well established as a clinical marker for diagnosis of alcohol abuse and considered to be more specific than other markers (for a recent review, see [30]). CDT has also been tried in forensic applications, but results here are less clear-cut. There appears to be a severe problem with in vitro decay during storage [14], cut-off diagnostic levels have to be set considerably higher than in clinical materials to give reasonable specificity [31] and different methods of CDT analysis (i.e. isoelectric focusing and immunoassay) give different relative cut-off values [31]. CDT has also been analysed in vitreous humour, but a separation between alcoholics and controls was inconclusive [32]. Due to the interpretative and technical difficulties listed, CDT was not used in this material. Further studies are needed to delineate the advantages of CDT in relation to other markers.

In conclusion, measurements of phosphatidylethanol (PEth) yield improved information on previous alcohol abuse in forensic autopsy cases, especially where other indications may be absent. Further studies are needed to compare PEth with other markers, such as ethyl glucuronide and CDT and/or to evaluate a combination of them [29].

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