Can PEth Be Detected with a Cutoff of 20 ng/mL after Single Alcohol Consumption?

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Abstract

Phosphatidylethanol (PEth) can be determined in capillary blood collected as dried blood spots (DBS) and is a promising direct alcohol biomarker for determination of drinking habits. Its use for abstinence monitoring needs to be evaluated. Studies with patients undergoing alcohol withdrawal have shown that elimination of PEth can take up to two months. For the determination of PEth 16:0/18:1, a cutoff of 20 ng/mL has been agreed upon in the major US laboratories. However, it is not yet clear what minimum blood alcohol concentrations (BACs) have to be achieved by a single drinking episode to result in PEth concentrations above this cutoff after previous long-term abstinence. To determine whether low drinking amounts can result in a positive PEth concentration above 20 ng/mL, we recruited 12 participants ("social" drinkers). After four weeks of abstinence, alcohol was consumed at two separate drinking events with target BACs of 0.5 and 0.3 g/kg, resulting in maximum BACs in the ranges of 0.30–0.63 g/kg and 0.10–0.28 g/kg, respectively. Capillary blood was collected at different time points of the drinking experiment and PEth was extracted from dried blood spots (DBS) and analyzed by liquid chromatography–tandem mass spectrometry. Despite drinking doses up to 0.58 g ethanol per kg body weight and reaching BACs of up to 0.63 g/kg, PEth 16:0/18:1 and PEth 16:0/18:2 could not be detected at or above the 20 ng/mL cutoff in any participant at any time after the drinking events. We conclude that after long-term abstinence the cutoff of 20 ng/ml for single alcohol consumption leading to blood alcohol concentrations up to 0.63 g/kg is not exceeded.

Keywords: phosphatidylethanol (PEth); drinking study; alcohol biomarker; abstinence monitoring; ethanol

Introduction

Driving under the influence of alcohol remains a major problem for traffic safety worldwide. After revocation a driver's license due to drunk driving, abstinence monitoring for up to one year is required for the assessment of driving aptitude before a driver's license is regranted (in Germany and in Switzerland). Furthermore, abstinence monitoring is used in alcohol withdrawal therapy. Both direct and indirect alcohol biomarkers exist for the assessment of alcohol consumption. Whereas indirect alcohol biomarkers such as gamma-glutamyltransferase (GGT), mean red blood cell corpuscular volume (MCV) and carbohydrate-deficient transferrin (CDT) can be influenced by underlying chronic diseases, direct alcohol biomarkers such as ethyl glucuronide (EtG) and ethyl sulfate (EtS) are formed enzymatically only in the presence of ethanol. Direct alcohol biomarkers have an advantage over indirect ones due to their sensitivity and specificity [1].

Phosphatidylethanol (PEth) is a promising direct biomarker for alcohol consumption, which gained popularity in the last decade. PEth is a group of phospholipids formed in cell membranes of various tissues as product of the reaction of phosphatidylcholines with ethanol, catalyzed by phospholipase D, and is only formed as long as ethanol is present in the body [2,3]. More than 48 analogues with various fatty acid chains are known, with PEth 16:0/18:1 and PEth 16:0/18:2 being the predominant molecular species [4-6]. In comparison to the indirect alcohol biomarker CDT, PEth was shown to have superior sensitivity, allowing the detection of alcohol consumption at lower concentrations [7-10]. Whereas biological variables such as higher hemoglobin, advanced liver fibrosis and higher BMI have been associated with PEth sensitivity, no association between sex or age and PEth sensitivity has been found, making it a broadly applicable marker [11]. According to Faller et al., long-term stability in liquid blood samples is only guaranteed when stored at –80°C degrees, while Skråstad and colleagues have shown that liquid blood samples are

stable for 28 days even at ambient temperature (mean 22.1°C) [12,13]. Therefore, and due to the better shipping possibilities, dried blood spots (DBS) were used in this study. The PEth concentrations in liquid blood samples and DBS are comparable [14,15]; in addition, the drying process causes inactivation of the enzymes and any ethanol present evaporates, resulting in increased stability [16]. A mean half-life of 7.8 ± 3.3 [SD] days and 6.4 ± 5.0 [SD] days is suggested for PEth 16:0/18:1 and 16:0/18:2, respectively [17]. Recently, in a study by Luginbühl et al. with patients undergoing alcohol withdrawal treatment, a biphasic elimination kinetic was found with an initial elimination half-life of 2.4 and 2.3 days and a terminal elimination half-life of 13.6 and 10.2 days for 16:0/18:1 and 16:0/18:2, respectively [14]. In this study, it was possible to detect PEth up to 2 month after cessation of alcohol abuse.

PEth has mostly been used to detect moderate and heavy drinking in the past [18,19]. However, in recent years, it has found broader application as alcohol biomarker in detoxification programs [20], as diagnostic tool in the antenatal setting [21,22], as screening tool to detect harmful alcohol consumption [23], and in quantification of alcohol consumption in adolescents and young adults [24]. PEth has been found to be useful for detecting recent moderate alcohol consumption within a three-month period and for monitoring abstinence in driving aptitude assessments [25,26]. Thresholds to reject abstinence are proposed at a PEth 16:0/18:1 concentration of 20 ng/mL in the US and 35 ng/mL (0.05 µmol/L) in Sweden, whereas 210 ng/mL indicates chronically elevated alcohol consumption [27-29]. With detection windows up to several weeks after excessive alcohol consumption, PEth might be used in abstinence monitoring.

EtG and EtS are formed enzymatically as by-products during alcohol metabolism by glucuronidation and by sulfation, respectively [30]. EtG and EtS in urine are commonly used to detect previous alcohol consumption and are detectable in urine for less than one

day to a few days, depending on the drinking amount [31-35]. For abstinence monitoring with EtG in urine (uEtG), the time between announcing an obligatory urine sampling and performing the sampling must not exceed 24 hours in German programs for abstinence monitoring.

Recent studies have shown that PEth formation occurs after both multiple and single consumption of small amounts of alcohol resulting in a BAC of up to 1 g/kg [36,37]. Javors and colleagues demonstrated the formation of PEth after single doses of either 0.25 g or 0.5 g of ethanol per kg body weight, with maximum PEth concentrations between 90 and 120 minutes post alcohol consumption [38]. The authors used a cutoff of 5 ng/mL, while in the US a cutoff of 20 ng/mL has been agreed on [27,28]. The same researchers showed proportional increases in PEth concentration after single alcohol consumption of 0.4 g/kg and 0.8 g/kg. However, PEth concentrations that were above the quantification limit in most participants even before the onset of alcohol consumption, indicating that the required abstinence period was not sufficient [17]. In our study we therefore used a four week abstinence period and a cutoff of 20 ng/mL for each PEth analogue chosen. This quantification limit allowed us to use a previously published, fully validated method with automated DBS extraction [39].

The aim of this study is to determine whether consumption of low amounts of ethanol after long-term abstinence results in PEth concentrations above the proposed cutoff of 20 ng/mL. Therefore, we conducted drinking experiments for the evaluation of dose-related effects of single alcohol consumption at the target BACs of 0.5 g/kg and 0.3 g/kg at intervals of 10 days on the formation and elimination of PEth 16:0/18:1 and PEth 16:0/18:2.

Materials and methods

Ethical vote

Approval was granted by the Ethics Committee of the Albert-Ludwig-University of Freiburg, Freiburg, Germany in 2021 (20-1243). All procedures performed were in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Participants

Twelve participants were recruited at the University of Freiburg, Germany. They had to be at least 20 years old and willing to abstain from alcohol four weeks prior to and until the end of the experiment. Exclusion criteria were alcohol use disorder history, major cardiac, pulmonary, renal, hepatic, neurologic or psychiatric problems, as well as diabetes or pregnancy. The average amount of alcohol consumed per week was determined on the basis of self-reporting by the subjects using a standardized questionnaire. As is customary in Germany and Switzerland, a standard drink was defined as a beverage containing 10 g of alcohol [40]. The descriptive data of the participants are shown in Table I.

Experimental design

The study included a pre-study interview for each participant in which information about the study and risks were provided, and initial venous blood samples were collected for analysis of CDT, GGT and MCV in order to reveal chronic excessive alcohol-consumption or to detect a major underlying disease. DBS were obtained directly after collection from the venous blood samples and analyzed for PEth to exclude those with excessive alcohol consumption. After participants had given their written consent, a four-

weeks-period of alcohol abstinence started, followed by two drinking events (10 days apart) with different target BACs (0.5 g/kg vs. 0.3 g/kg). The abstinent phase between the two drinking events was only 10 days. Once before each drinking event urine samples were analyzed for EtG to support participants' report of abstinence. From this urine sample, a pregnancy test was performed in female participants prior to drinking. DBS samples were required several times during the study for the analysis of PEth. One sample was collected at the pre-study interview (from venous blood) and a total of 12 DBS samples were collected per drinking event (by sampling from the fingertip): before drinking at 9 am (1), 30 minutes (first drinking event) / 15 minutes (second drinking event) after drinking (2), after individual ethanol elimination as described below (3), on the same day at 9 pm (4), on the following three days at 9 am and 9 pm (5–10) and on the following two days at 9 am (11–12). The drinking events were accompanied by regular measurements of breath alcohol concentrations (BrAC) every 15 minutes. The experimental time ended for the participants 5 days after the second drinking event. During this entire period (about seven weeks), participants were required to abstain from any alcohol consumption apart from the drinking events. A summary of the time sequence of the experiment is shown in Figure 1.

Study interventions such as recruiting participants, drinking events and sample collection as well as measurement of BAC took place at the Institute of Forensic Medicine Freiburg, Germany, from February to April 2021. The analysis of the clinical biomarkers CDT, GGT, MCV was performed at the Institute for Clinical Chemistry and Laboratory Medicine (Medical Center, University of Freiburg); analysis of EtG in urine and PEth in DBS samples was performed at the Institute of Forensic Medicine Bern, Switzerland.

Drinking events

For the sake of comparability, all drinking events started at 9 am. In the first drinking event, the amount of consumed alcohol (ABSOLUT Vodka, 40% ethanol, THE ABSOLUT COMPANY AB, Sweden) was supposed to lead to a BAC of 0.5 g/kg, in the second to a BAC of 0.3 g/kg. The individual amount of vodka was calculated by the Widmark Formula [41] with a resorption deficit defined as 20%. The distribution coefficient was determined by sex: 0.6 for female, 0.7 for male participants. BAC was measured in venous blood samples 30 minutes after the end of drinking in the first drinking event and 15 minutes after the end of drinking in the second drinking event to capture the maximum blood alcohol concentration. After the first BAC concentrations were obtained, it was found that the achieved BAC tended to be too low. Therefore, approximately 20% more alcohol was planned in subsequent experiments in order to achieve the target BACs. The drinking amounts are listed in Table II. Alcohol intake could be facilitated by mixing with soft drinks (e.g., bitter lemon, passion fruit juice, orange juice). Drinking duration was expected not to exceed 30 minutes.

Every 15 minutes breath alcohol was determined by an alcohol breath analyzer Alcotest 6510 (Drägerwerk AG & Co. KGaA, Lübeck, Germany) starting 15 minutes after the end of drinking. Drinking events including elimination phase ended 30 minutes after having reached a BrAC equal to or below 0.05 mg/L (duration of first drinking event: mean = 4.6 h, SD = 0.80 h; duration of second drinking event: mean = 2.7 h, SD = 0.46 h). Alcohol intake was accompanied at all times by a medically trained supervisor.

Sampling

Capillary blood for DBS was collected from fingertips. After the sampling area had been sanitized with ethanol-free disinfectant, the fingertip was punctured with a Safe-T-

Pro-Plus lancet from Accu-Chek (Roche Diabetes Care, Rotkreuz, Switzerland). The released blood drop was applied to a filter paper card (GreenCheck DBSC, Protzek, Lörrach, Germany). Four blood spots were generated on each filter paper card. At the first drinking event, subjects were trained to collect DBS on their own. DBS were prepared from 20 µL of venous blood sampled at the pre-study interview and after the end of drinking (30 and 15 minutes in the first and second drinking experiment, respectively). All other DBS samples were prepared from capillary blood directly on DBS cards. The DBS were allowed to dry at room temperature for two hours and then stored in a minigrip bag with a silica desiccant gel packet at –20°C in a freezer until they were delivered to the Institute of Forensic Medicine, Bern, for analysis.

Sample preparation and instrumental analysis of BAC, PEth and EtG

The analysis of BAC was performed from venous blood samples by a validated headspace gas chromatography method with flame ionization detection (HS-GC–FID) at the Institute of Forensic Medicine Freiburg, Germany. The blood samples were centrifuged for 15 min and 100 µL supernatant was pipetted with 500 µL ter-butanol solution (internal standard) into a 20 mL headspace vial. According to forensic guidelines, samples were analyzed twice on each of two GC–FID systems, with a Clarus 580 coupled to a TurboMatrix headspace sampler (Perkin Elmer, Rodgau, Germany) with a Rtx®-BAC Plus 1 column (30 m x 0.32 mm x 1.8 µm, Restek, Bad Homburg, Germany) and with a Clarus 680 coupled to a TurboMatrix headspace sampler 40 Trap (Perkin Elmer, Rodgau, Germany) with a Rtx®-502.2 column (60 m x 0.53 mm x 3 µm, Restek, Bad Homburg, Germany) [42].

Analysis of capillary DBS and DBS obtained from venous blood for PEth was carried out at the Institute of Forensic Medicine in Bern by LC–MS-MS, as described elsewhere [39]. A fully automated DBS extraction was performed using a CAMAG DBS-MS

500 instrument (CAMAG, Murten, Switzerland). The mass spectrometer used was a 5500 QTRAP (Sciex, Toronto, ON, Canada). The fully validated method with a limit of detection (LOD) of 10 ng/mL and a lower limit of quantification (LLOQ) of 20 ng/mL has been published recently [43]. Accordingly, these limits were applied for the study. EtG was analyzed by LC–MS-MS with a 3200 QTrap system (Sciex) [44]. The common accepted EtG cutoff of 100 ng/mL was applied [45].

Results

Blood alcohol concentrations are listed in Table II and plotted in Figure 2. For PEth 16:0/18:1 and PEth 16:0/18:2, no concentrations above the proposed cutoff and LLOQ of 20 ng/mL could be detected. However, the PEth concentrations of subjects 3 and 7 were below the LLOQ but not below the LOD (10 ng/ml) before the first drinking event. After drinking, the concentrations remained in this range. Thus, all concentrations of measured PEth analogues were below the LLOQ, and the results were reported as < 20 ng/mL. The mean maximum BrAC was 0.22 (SD = 0.05) mg/L and 0.12 (SD = 0.03) mg/L at BAC target 0.5 g/kg and 0.3 g/kg, respectively. Maximum BrACs at the first drinking event were measured 15 minutes (n = 6), 30 minutes (n = 2) and 45 minutes (n = 4) after the end of drinking. At the second drinking event, maximum BrACs were measured 15 minutes (n = 9), 30 minutes (n = 2) and 45 minutes (n = 1) after the end of drinking. Final BrACs equal to or below 0.05 mg/L were reached in both drinking events in 3.04 h (SD = 0.94) and 1.6 h (SD = 0.47) after the end of drinking.

Indirect alcohol biomarker analysis revealed normal concentrations for GGT, MCV, and CDT in all subjects, indicating no excessive alcohol consumption. EtG concentrations were determined to support participants´ reports of abstinence before each drinking event. All but one subject were below the EtG cutoff; in subject 5, in the second drinking

event the concentration of EtG in urine taken prior to the start of drinking was 320 ng/mL (170 ng/mL when normalized to a creatinine concentration of 100 mg/dL).

Discussion

This study examined whether measurable PEth concentrations are generated in subjects after a single consumption of alcohol with a target BAC of 0.5 and 0.3 g/kg, respectively. In addition, it was planned to observe the elimination of the two PEth analogues 16:0/18:1 and 16:0/18:2 over 5 days in order to be able to make a statement about the detection time. However, none of the 12 subjects produced measurable PEth concentrations above the LLOQ of 20 ng/mL after consuming either amount of alcohol. Subjects 3 and 7 began the first drinking event with values between the LOD and the LLOQ. However, these two subjects also remained below the cutoff of 20 ng/mL after drinking. The fact that different cutoffs were used in our study (20 ng/mL) and in the study of Javors et al. (5 ng/mL), makes is difficult to compare the results [38]. However, with only 7 days of abstinence prior to alcohol consumption, not all study participants in the cited study maintained a sufficiently long abstinence period. This resulted in PEth concentrations between 12 and 347 ng/mL being detectable in 22 of a total of 27 subjects prior to the start of the drinking experiment. Furthermore, PEth was determined in liquid blood that was initially stored at 4°C for up to 24 hours after collection and only then frozen at –80°C until analysis. Therefore, the alcohol present in the samples may have led to post-sampling formation of low concentrations of PEth.

In contrast to previous studies, a strength of our study is that all participants started the drinking experiments with a negative PEth result below the applied cutoff [17,36,38,46]. The participants' drinking habits were surveyed during the initial interview and measured indirect alcohol biomarkers did not indicate excessive alcohol use. Study

participants were requested to maintain four weeks of abstinence prior to the first drinking event, as well as between each drinking event, to ensure a negative PEth result (< 20 ng/mL) at the start of each drinking event. Adherence to abstinence was additionally monitored by PEth determination in blood samples and with EtG samples obtained approximately 15 minutes before each drinking event. All urine samples collected prior to the drinking events were negative for EtG except one (subject 5). The positive EtG concentration of subject 5 before the second drinking event indicates the uptake of ethanol and could have resulted from the consumption of so-called non-alcoholic beer or other beverages, which may have a very little content of alcohol of 5 g/L, and other sources of ethanol traces cannot be excluded [47]. Another reason for the positive EtG concentration could be that the participant has not adhered to abstinence and has consumed an alcoholic beverage. Nevertheless, in subject 5, as in all other subjects, no positive PEth concentrations above 20 ng/mL were detected - neither before nor after the drinking event. With nonquantifiable pre-drinking PEth concentrations on drinking days and by testing for the indirect alcohol markers CDT, GGT and MCV, which were in normal ranges, excessive drinking before the experiment could be excluded in all participants. Thus, all subjects could be included in the evaluation.

The blood alcohol concentrations determined after 30 minutes in the first drinking event and after 15 minutes in the second one do not necessarily correspond to the maximum alcohol blood concentrations reached. By measuring the BrAC every 15 minutes after the end of drinking, the highest BrAC were found at later time points (45–60 min after the end of drinking) in 42% in the first drinking event and in 33% in the second drinking event. However, it should be mentioned that the blood and breath ethanol concentrations are only comparable to a limited extend. Two participants who consumed rapidly reached the maximum concentrations the latest, participant 11 after 60 minutes in the first drinking

event and participant 12 after 45 minutes in the second drinking event. Maximum breath alcohol concentration indicated concentrations of 0.13–0.32 mg/L (mean: 0.21 mg/L) in the first drinking event with a target BAC of 0.5 g/kg and concentrations of 0.08–0.17 mg/L (mean: 0.12 mg/L) in the second drinking experiment with a target BAC of 0.3 g/kg. However, the highest measured blood alcohol concentration is consistent with the highest measured BrAC and therefore it can be concluded that the PEth concentration remained below 20 ng/mL even after the highest achieved BAC of 0.63 g/kg.

A limitation of the study was that there were no requirements regarding food intake before drinking. A full stomach may delay ethanol absorption, so that the measured BAC after drinking may thus appear lower than it would have been without food intake at that time. Another limitation of the study, especially if a general statement would be made, is the limited number of participants. However, an insight into the formation of PEth can be gained even with a smaller number of study participants. Considering the fact that only two subjects started the drinking events with PEth concentrations between LOD and LLOQ, it cannot be excluded that further subjects in this particular group would exceed the limit of 20 ng/mL after drinking.

Conclusions

We have demonstrated that single alcohol consumption with maximum blood alcohol concentrations of 0.1 g/kg up to 0.63 g/kg did not result in detection of PEth concentrations above 20 ng/mL, although two subjects started with concentrations between 10 and 20 ng/mL (LOD and LLOQ, respectively). PEth 16:0/18:1 and PEth 16:0/18:2, each with a cutoff of 20 ng/mL, therefore do not appear to be suitable to unequivocally detect a single alcohol consumption with doses of up to 0.58 g/kg body weight after four weeks of abstinence in subjects who had no previous excessive alcohol consumption.

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Data Availability Statements

The data underlying this article are available in the article.

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figure captions

Figure 1. Time schedule of the experiment with corresponding samples.

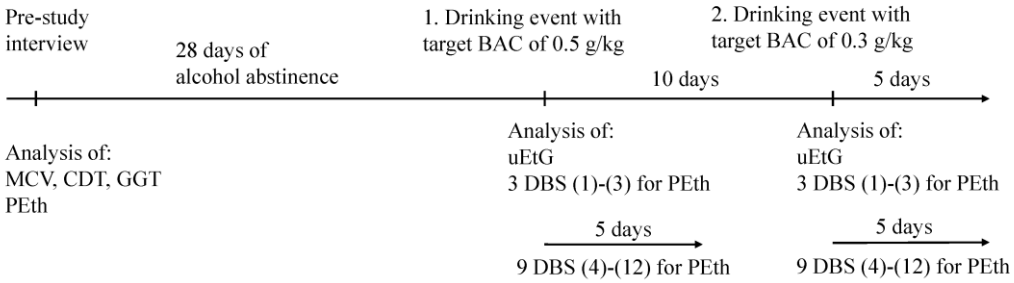

Figure 2. BACs [g/kg] obtained in the first drinking event (0.5 g/kg; solid line) and the second drinking event (0.3 g/kg; dashed line) are shown.

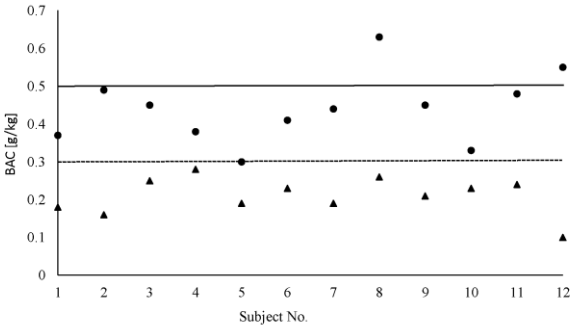

Table I. Details of the Volunteers Participating in the Study

Subject	Sex	Age [years]	Weight [kg]	BMI	Weekly Alcohol Intake[a]
1	f	24	50	17.3	1.5
2	f	24	61	22.4	3
3	f	26	70	23.9	5
4	f	23	59	23.0	2
5	f	25	56	21.3	2.5
6	f	23	56	21.3	6
7	m	27	90	26.6	10
8	m	24	67	20.5	2
9	m	25	73	20.9	1.5
10	m	27	61	19.0	6
11	m	26	77	22.5	4
12	m	23	67	22.4	4.5
Mean (SD)		24.75 (1.48)	65.56 (10.67)	21.77 (2.36)	4 (2.50)

[a] Number of standard drinks with 10 g of alcohol.

Table II. Drinking Details of the Participants

Subject	Target BAC [g/kg]	Pure ethanol calculated [g]	Dose [g/kg][b]	Amount of 40% vodka given [ml]	Drinking duration [min]	BAC [g/kg]
1	0.5	18.8	0.38	61	25	0.37
	0.3[a]	13.8	0.28	43	16	0.18
2	0.5	22.9	0.38	71	5	0.49
	0.3[a]	17.2	0.28	54	5	0.16

3	0.5[a]	30.2	0.43	94	26	0.45
	0.3[a]	19.7	0.28	62	10	0.25
4	0.5[a]	25.4	0.43	80	15	0.38
	0.3[a]	16.6	0.28	52	14	0.28
5	0.5[a]	28.2	0.50	88	38[c]	0.30
	0.3[a]	18.4	0.33	57	21	0.19
6	0.5[a]	28.2	0.50	88	5	0.41
	0.3[a]	18.4	0.33	57	1	0.23
7	0.5[a]	45.3	0.50	142	26	0.44
	0.3[a]	29.5	0.33	92	11	0.19
8	0.5[a]	38.5	0.57	120	5	0.63
	0.3[a]	25.1	0.37	79	23	0.26
9	0.5[a]	42.0	0.58	131	5	0.45
	0.3[a]	27.4	0.38	86	2	0.21
10	0.5[a]	30.7	0.50	96	16	0.33
	0.3[a]	20.0	0.33	63	17	0.23
11	0.5[a]	44.3	0.58	138	6	0.48
	0.3[a]	28.9	0.38	90	1	0.24
12	0.5[a]	38.5	0.57	120	6	0.55
	0.3[a]	25.1	0.37	79	1	0.1
Mean (SD)	0.5	32.8 (8.74)	0.49 (0.07)	102 (25.8)	14.8 (11.4)	0.44 (0.09)
	0.3	21.7 (5.05)	0.33 (0.04)	67.8 (15.8)	10.2 (8.11)	0.21 (0.05)

[a] Original calculated dose was adjusted by adding 20 % more alcohol.

[b] Dose in grams of ethanol per kg body weight.

[c] Drinking period was slightly longer (38 min instead of 30 min).