HHS Public Access

Author manuscript

Alcohol. Author manuscript; available in PMC 2023 January 09.

Published in final edited form as:

Alcohol. 2022 February ; 98: 51–54. doi:10.1016/j.alcohol.2021.11.001.

Comparison of automated determination of phosphatidylethanol (PEth) in dried blood spots (DBS) with previous manual processing and testing

Robin Fatch[a], **Marc Luginbühl**[b], **Debbie M Cheng**[c], **Stefan Gaugler**[b], **Nneka I Emenyonu**[a], **Christine Ngabirano**[d], **Julian Adong**[d], **Winnie R Muyindike**[d], **Jeffrey H Samet**[c,e], **Kendall Bryant**[f], **Judith A Hahn**[a,g]

[a]Division of HIV, Infectious Diseases, and Global Medicine, Department of Medicine, University of California, San Francisco, CA, USA.

[b]CAMAG DBS Laboratory, Muttenz, Switzerland

[c]Boston University School of Public Health, Boston, MA, USA

[d]Mbarara University of Science and Technology, Mbarara, Uganda

[e]Boston University School of Medicine; Boston Medical Center, Boston, MA, USA

[f]National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD, USA

[g]Department of Epidemiology and Biostatistics, University of California, San Francisco, CA, USA.

Abstract

Phosphatidylethanol (PEth) is a sensitive and specific biomarker of alcohol consumption in the prior 2–3 weeks. Standard, manual PEth testing using dried blood spots (DBS) is a multi-step time-consuming process. A novel, automated processing and testing method has been developed to decrease DBS processing and testing time. We conducted automated testing, using regiosimerically pure PEth reference material, on randomly selected DBS which had previously been tested via manual methods and then stored for 3–6 years at at −80°C, to compare the results (PEth 16:0/18:1 homologue). We chose samples for re-testing using categories found in the literature as follows: (1) PEth <20 ng/mL; (2) PEth 20–200 ng/mL; (3) PEth >200–1000 ng/mL; (4) PEth >1000 ng/mL. We calculated agreement between the categories using the weighted kappa statistic (n=49 DBS). We quantified agreement between continuous measures using the intraclass correlation coefficient (ICC), and further described the relationship between variables using Spearman correlation. The median PEth result was 155 ng/mL (interquartile range [IQR]: 1–1312 ng/mL) via automated methods and 98.8 ng/mL (IQR: 10.2–625.0 ng/mL) via manual methods. The weighted kappa comparing the automated to manual PEth results was 0.76 (95% Confidence Interval (CI): 0.66–0.86). The ICC was 0.69 (95% CI: 0.54–0.79), and the Spearman correlation was 0.98 (95% CI: 0.95–0.99). While the new methods yielded somewhat higher PEth values, we found good to excellent agreement between clinically relevant PEth categories.

Corresponding author: Judith A. Hahn, judy.hahn@ucsf.edu, 550 16th Street, Box 1224, Floor 03, Room 3402, San Francisco, CA 94158, 415-476-5815.

Automated DBS processing and testing using new reference standards are promising methods for PEth testing.

Keywords

phosphatidylethanol; PEth; alcohol biomarker; dried blood spots; automated; LC-MS/MS

Introduction

Phosphatidylethanol (PEth) is a sensitive and specific direct biomarker of alcohol consumption in the prior 2–3 weeks (Wurst et al., 2015). Objective measures of alcohol use are critical for medical care, alcohol treatment, and alcohol research, as self-reported measures are imperfect, often subject to issues including recall bias and socially desirable reporting. PEth has been utilized in research studies to detect under-report of alcohol use (Bajunirwe et al., 2014; Magidson et al., 2019; Muyindike et al., 2017), examine HIV morbidity and mortality (Eyawo et al., 2018; Hahn et al., 2018), as outcomes for trials of alcohol interventions (Edelman et al., 2019; Walther et al., 2015; Wang et al., 2017), and in clinical practice, primarily in Europe (Ulwelling & Smith, 2018). PEth is formed within red blood cells, and can be detected in whole blood and in dried blood spots (DBS). DBS have several advantages over whole blood, including stability, lack of in vitro formation of PEth, ease of transport, and ease of collection (Kummer et al., 2016; Wagner, Tonoli, Varesio, & Hopfgartner, 2016).

Standard, manual PEth testing consists of manual punching of DBS cards, eluting and incubating the blood, and liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Jones, Jones, Plate, & Lewis, 2011). A novel automated PEth testing method was developed by Luginbühl et al (Luginbühl, Gaugler, & Weinmann, 2019), which consists of using fully automated DBS sample preparation, linked to an online solid phase extraction LC-MS/MS. This testing has shown a high correlation (0.97) with manual DBS extraction (and the same subsequent processing) in a sample of 28 persons with alcohol use disorder. The automated DBS sample preparation eliminates the manual DBS sample preparation time of about 3 hours for 48 DBS samples (the maximum centrifuge capacity for manual preparation). Instead, the fully automated sample extraction occurs as part of the LC-MS/MS run. In addition, the field has identified the need for regioisomerically pure PEth reference material to improve the reliability of the PEth analysis (Luginbühl et al., 2020).

The objective of this analysis was to compare the PEth results previously obtained from manual processing and testing at an external laboratory to those obtained from automated processing and testing using regioisomerically pure PEth reference material in a sample of research participants with a range of drinking. DBS samples, stored after the initial manual PEth analysis in a freezer at −80 °C for 3 to 6 years, were reanalyzed using fully automated PEth determination.

Materials and Methods

Study sample and procedures

The samples for this analysis were collected as part of the Alcohol Drinking Effects Prior to Treatment (ADEPT) study, a longitudinal cohort study that is part of the Uganda Russia Boston Alcohol Network for Alcohol Research Collaboration of HIV/AIDS (URBAN ARCH) Consortium. The ADEPT study recruited participants from the Immune Suppression Syndrome (ISS) Clinic in Mbarara, Uganda from 2011 to 2014 to better understand alcohol's impact on HIV disease progression (Hahn, et al., 2018). Study eligibility criteria included: age ≥ 18 years old, HIV infection, fluency in English or Runyankole (the local language), living within the study catchment area, and not yet receiving antiretroviral therapy (ART). The study catchment area was originally within 60km of the clinic; in the final year of the study this was expanded to 120km for males, with the hopes of increasing enrollment of men who consume alcohol. Prior to March 2014, the CD4 cell cutoff for ART initiation at the clinic was <350 cells/mm^3; after which, the cutoff changed to <500 cells/mm^3. ADEPT participants (n=447) were enrolled and completed study visits at baseline and every 6 months, until they were eligible to initiate ART or the study concluded (December 2015). Those eligible to initiate ART received a final exit visit prior to initiation.

All study visits included a structured interviewer-administered questionnaire and venous blood draw. Whole blood was pipetted onto Whatman 903 protein saver DBS cards later that day and stored at −80°C. The study activities were approved by the Ethics review boards of Mbarara University of Science and Technology, University of California San Francisco, Boston University, and Boston Medical Center.

For this analysis, we randomly selected 50 DBS cards from 4 gender-balanced subgroups from the 446 ADEPT study baseline visits, based on the previous manual PEth levels (testing described below): (1) PEth <20 ng/mL (n=15); (2) PEth 20–200 ng/mL (n=15); (3) PEth >200–1000 ng/mL (n=15); (4) PEth >1000 ng/mL (n=5). A cutoff of <20 ng/mL was chosen for group 1 as this was the lower limit of quantification (LLQ) for the automated PEth testing, and the cutoff of 200 was roughly consistent with the >210 ng/mL cutoff for excessive drinking (Helander and Hansson, 2013).

Manual PEth testing

The DBS cards were shipped in batches at room temperature to a commercial laboratory (United States Drug Testing Laboratory [USDTL], Des Plaines, IL) for PEth testing. DBS cards were tested for the PEth 16:0/18:1 homologue as part of the ADEPT study in batches at USDTL using previously published methods (Jones, et al., 2011), between 2013–2016. The lower limit of detection was 2 ng/mL and the LLQ was 8 ng/mL. The DBS cards minus the punches used for the PEth analysis were then re-stored in a specimen repository at −80°C.

Automated PEth testing

In November 2019, the sample of 50 DBS cards (sampling described above) were sent to the CAMAG DBS Laboratory (Muttenz, Switzerland). Operators cut the cards to size because

slightly smaller dimensions were needed and determined PEth 16:0/18:1 and PEth 16:0/18:2 levels, using the CAMAG DBS-MS 500 system linked to online LC-MS/MS (Shimadzu 8050), adapted as previously described (Luginbühl, et al., 2019).

Analysis

All results discussed here are for the PEth homologue 16:0/18:1, as this was the homologue tested via both methods. We describe PEth levels using medians and interquartile ranges (IQR). We calculated a weighted kappa statistic to describe agreement between the PEth groups based on the 4 ordinal selection categories.

We also compared the continuous PEth results. Results below the LLQ (n=16) were set to 1 ng/mL; results from automated testing >1500 ng/mL (n=10) were outside the window of calibration for this method, and were set to the median of these values. We calculated the intraclass correlation coefficient to quantify agreement using the ICC (3, 1) form (Shrout & Fleiss, 1979). To further describe the relationship between the two sets of results, we also calculated the Spearman correlation and bootstrap percentile-based 95% confidence intervals (CI).

Stata 14.2 (StataCorp, College Station, TX, USA), SAS 9.4 (SAS Institute Inc., Cary, NC, USA), and R 4.0.0 (R Core Team, Vienna, Austria) were used for these analyses.

Results

Fifty samples were selected for automated testing; one sample did not have sufficient blood remaining on the DBS card and was thus excluded. Twenty-five (51%) DBS cards were from female participants, and the median age was 32 years (IQR: 28–40).

The median manual PEth testing result was 98.8 ng/mL (IQR: 10.2–625.0) and the median automated PEth result was 155 ng/mL (IQR: 1–1312). We found excellent agreement of the pre-determined categories (weighted kappa statistic=0.76, 95% CI: 0.66–0.86). Table 1 shows the comparison of the manual and automated PEth results. The highest two subgroups (>200–1000 ng/mL and >1000 ng/mL) accounted for most of the disagreement; when we pooled these two groups into one group (>200 ng/mL, near the cutoff for “excessive” alcohol use of 210 ng/mL (Helander & Hansson, 2013)), the weighted kappa statistic increased to 0.89 (95% CI: 0.80–0.98). We found good agreement between the continuous measures (ICC=0.69; 95%CI:0.54–0.79), and the Spearman correlation was 0.98 (95% CI: 0.95–0.99). PEth results obtained via the automated testing method appeared to be higher than those obtained via the manual method, as seen in Figure 1.

Discussion

Comparing PEth results obtained via two different testing methods, we found results via automated testing methods to be in good to excellent agreement with those obtained using manual methods over a broad range of PEth levels, using relevant groups and quantitative values. We additionally found a high correlation between the two analyses, similar to previous internal findings conducted on alcohol use disorder patients (Luginbühl, et al.,

2019). Some of the differences observed may be due to time elapsed between tests, differences in the analyte extraction methods, differences in the purity of the reference materials (Luginbühl, et al., 2020), or other reasons needing further exploration. Differences in the extraction process may contribute to differences in the PEth results, depending on the time of incubation for the manual extraction and the solvent used. Differences in the purity of the reference materials are likely the major contribution to differences in the PEth results. The automated testing used regioisomerically pure PEth reference material to improve the reliability of the PEth analysis (Luginbühl, et al., 2020), while the manual testing likely used the PEth m/z 701.5→255.2 transition from non-regioisomerically pure material for quantification (based on the ion ratio noted in the figure) (Jones, et al., 2011). When the reference material used is not 100% isomerically pure and the 255.2 transition is used as a quantifier, this may lead to underestimation of the actual concentration (Van Uytfanghe et al., 2020), and the PEth levels were higher using the new methods (Table 1 and Figure 1). While we acknowledge that these were different testing methods and thus some differences were expected, overall, we found relatively good agreement between PEth categories of clinical interest using the two methods.

There are some limitations to note. The sample size was small. Results that were <20 ng/mL or >1500 ng/mL via automated testing were outside the window of calibration due to the specifications of the LC-MS/MS used in the automated processing. We also note that while this automated method speeds up processing time and presumably lowers costs, the current machinery is most applicable to batched samples processed at sophisticated laboratories performing LC-MS/MS; therefore, it is not a point of care test.

In summary, the automated DBS processing and online PEth testing results had good to excellent agreement with previous manual testing. While results were not perfectly aligned, the clinically relevant PEth categories were similar, and automated methods have potential for decreasing the time and costs associated with PEth testing, especially for large batches of samples.

Funding:

This work was supported by grants from the National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism (K24AA022586, U01AA020776, U24AA020779).

Potential conflicts of interest:

Dr. Marc Luginbühl and Dr. Stefan Gaugler are employees of the DBS research laboratory at CAMAG (Muttenz, Switzerland), and did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors for this work. CAMAG donated the materials needed to do the automated testing.

Data availability:

The data underlying this article will be shared on reasonable request to the corresponding author.

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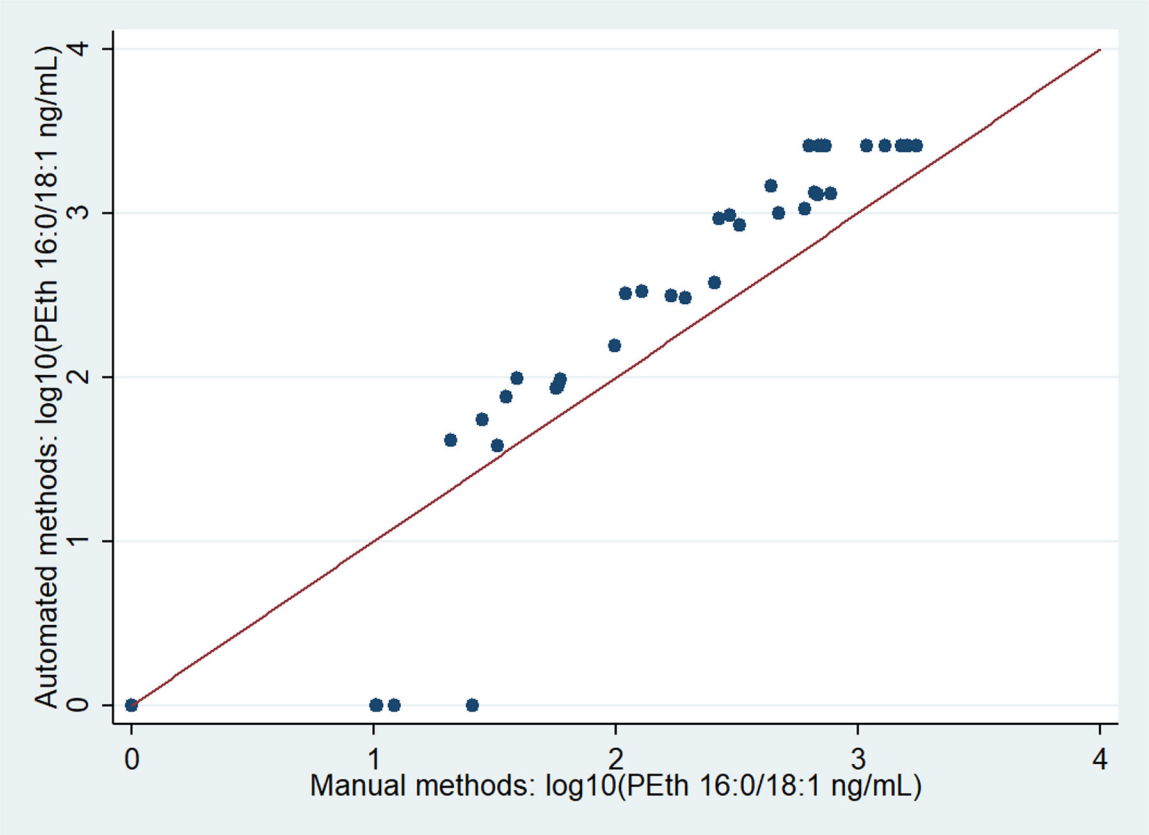

Figure 1.
Comparison of log10(phosphatidylethanol [PEth] 16:0/18:1 ng/mL) results obtained via manual and automated methods (n = 49).

Table 1.

Comparison of PEth results obtained via manual and automated processes (n = 49).

	Automated PEth processing				
Manual PETH processing	**<20 ng/mL**	**20–200 ng/mL**	**>200–1000 ng/mL**	**>1000 ng/mL**	**Total**
<20 ng/mL	15	0	0	0	15
20–200 ng/mL	1	9	4	0	14
>200–1000 ng/mL	0	0	5	10	15
>1000 ng/mL	0	0	0	5	5
Total	16	9	9	15	49