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Optimal Storage Conditions for Blood Samples, for Later Analysis in Criminal Prosecution Proceedings

Serpil Erdogan [1]*, Ozcan Erel [1,2], Orhan Sen[1], Gamze Avcioglu [1,2], Ceylan Bal[1,2], Pervin Baran[1]

1 Ataturk Training and Research Hospital, Department of Medical Biochemistry, Ankara, Turkey.
2 Yildirim Beyazit University, Faculty of Medicine, Department of Medical Biochemistry, Ankara, Turkey.

Abstract

Backround: Aim of this study was to demonstrate the stability of blood–ethanol concentration over a storage period. The precise storage time during which ethanol can be reliably measured in frozen blood samples is unknown.

Materials and Methods: In study A, samples were thawed and reanalyzed for ethanol content; their storage time since the first analysis ranged from one to 295 days. The percent difference between the first and second ethanol measurements was calculated as lost ethanol (%). For study B, the ethanol concentrations of 34 samples were measured within 180 days of, and at a randomly selected interval from, the working day.

Results: In study A, the calculated lost ethanol values ranged from 1% to 30%. There was no correlation between the calculated ethanol values and the measures on the second ethanol analysis day. A significant difference was detected between the two analyses in study B (p: 0.01).

Conclusions: It was concluded that blood samples to be analyzed for ethanol concentration can be stored at –80°C for many months, with no loss of concentration provided that analysis is undertaken as promptly as possible and blood samples are frozen as soon after collection as possible.

Key words: *Ethanol, stability, forensic medicine*

***Corresponding Author:** Serpil Erdoğan, Ataturk Egitim ve Arastirma Hastanesi, Tıbbi Biyokimya Bölümü, Üniversiteler Mh. Bilkent Cad. No:1, 06800 Çankaya, Ankara, Turkey **Phone:** +903122912525 **E-mail** drsturhan@yahoo.com
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Introduction

Ethanol, commonly called ethyl alcohol, is a product of the fermentation process. Alcoholic beverages comprise wines, beers, ciders, and several other alcoholic beverages that contain ethanol (1). As a frequently requested laboratory analysis, blood–ethanol concentration is measured generally for purposes such as diagnostics and therapeutic monitoring (2). Additionally, measurement of blood–ethanol concentration is an important analytical determination required to assess whether an offence has been committed (e.g., driving under the influence of alcohol) (3). The first legislation to ban drunk driving—in 1936, in Norway—has gone on to gain worldwide recognition, and legal boundaries have been defined with this legislation in mind (4, 5). In Australia, for a blood–ethanol concentration test, rules and standards have been defined for taking samples and transporting them to the laboratory; bearing in mind the probability of reanalyzing samples at a later date, it is suggested that samples be retained and preserved for at least 12 months (6).

Clinical laboratories, as highly complex facilities, have focused on quality control methods, and quality assessment studies have addressed the analytical phases of testing (7). However, a growing body of evidence that has accumulated in recent decades demonstrates that quality in clinical laboratories cannot be assured solely by focusing on purely analytical aspects (8). Accordingly, a lack of standardized procedures for the pre-analytical phase including patient preparation, specimen acquisition, handling, and subsequent storage account for up to 93% of the errors that occur during the diagnostic process (9). The precise length of storage time during which ethanol concentration in frozen blood samples can be reliably measured is unknown. To the best of our knowledge, very few studies report variations of blood–ethanol concentrations in plasma and serum when various pre-analytical settings are applied (2, 10-13). In 2008, Penetar and colleagues investigated the effect of storage conditions on plasma, serum, and whole-blood samples (12); meanwhile, in 2014, Kocak and colleagues evaluated the effects of different storage time intervals for blood samples (13). Interestingly, to quantify the evaporation rate of ethanol from uncapped samples, Saracevic et al. (2) investigated the effects of using unstoppered tubes on blood–ethanol concentration measurements taken at room temperature. Nevertheless, none of these studies investigated the effect of storage at –80°C, as well as storage over an extended period, on blood–ethanol concentration measurements.

Therefore, we investigated the effects of storage at –80°C and over a six-month period on blood–ethanol levels in collected blood samples. This study has two aims: to demonstrate the stability of blood–ethanol concentration over an eight-month period, versus time, and to investigate whether or not there were a difference between the ethanol concentrations measured before and after the six-month mark.

Materials and methods

This cross-sectional retrospective study was conducted in the Department of Clinical Biochemistry at the Ataturk Training and Research Hospital in Ankara, Turkey, with the approval of the local ethics committee.

Ethanol measurements had been performed with serum samples, with sampling done directly into serum vacuum tubes with a gel separator (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Following ethanol analysis, the remaining sera were stored at –80°C, given the possible need for retesting in support of a later criminal prosecution. In our hospital, 1,735 blood–ethanol concentrations were analyzed between July 2015 and February 2016; 1,559 of those blood–ethanol measurements were taken with a Cobas Integra 800 analyzer (Roche Diagnostics, Switzerland). Of those 1,559 samples, 287 were found to have blood–ethanol concentrations in excess of 20 mg/dL. Eighty-nine randomly selected samples were used in study A, and another 34 randomly selected samples were used in study B. Blood samples inappropriate for the current study—such as those that were lipemic or had been hemolyzed—were excluded.

The purpose of study A was to investigate sera stability at –80°C, over time. Eighty-nine frozen samples were randomly selected, and the results of the first blood–ethanol measurement were found to range from 28.5 to 395.2 mg/dL. The samples that had been first measured for ethanol were thawed and reanalyzed, one at a time. The calculated time interval since the first analysis ranged from one day to 295 days. The percent difference between the first and second ethanol measurements were calculated as lost ethanol (%) and residual ethanol (%), as per formulas 1 and 2, respectively.

Formula 1: Lost ethanol (%) = 100 × (Result of first ethanol measurement – Result of second ethanol measurement) / Result of first ethanol measurement

Formula 2: Residual ethanol (%) = 100 – lost ethanol (%)

Thereafter, we undertook a correlation analysis between the two blood–ethanol concentration values, for the 89 samples analyzed after 1–295 days and the second ethanol analysis day.

For study B, to demonstrate whether or not there was a difference between the concentration before and after six months of storage, we randomly selected 34 samples for which ethanol concentrations had been measured within 180 days of the working day. Over a 16-day period, each sample fulfilled 180 days after the first analysis was thawed and reanalyzed. Ethanol concentrations from both analyses were compared.

All of the ethanol analyses used in studies A and B were executed using a Cobas Integra 800 analyzer with original Roche reagents and the enzymatic alcohol dehydrogenase (ADH) method. Because repeated freezing and thawing should be avoided, the samples used in both studies were not identical.

Statistical analyses

Statistical analyses were performed using PASW Statistics 20 software. Normality of distribution was evaluated using a Kolmogorov–Smirnov test. Comparisons of variables with a normal distribution were made using a paired t-test, and values are provided as mean ± standard deviation. Correlation between the results of the first and second ethanol analyses was evaluated with a Pearson test, given the normality of distribution of these parameters. A p-value of less than 0.05 was considered indicative of statistical significance.

Results

Results of Study A

For study A, 89 samples were analyzed within 1–295 days, with a mean period of 126.7 ± 66.22 days (minimum one day, maximum 295 days). The first ethanol concentration of these samples ranged from 28.5 to 395.2 mg/dL (164.48 ± 79.89 mg/dL), and the second ethanol concentration of these samples ranged from 25.7 to 354.7 mg/dL (139.67 ± 67.31 mg/dL). The calculated lost ethanol values therefore ranged from 1% to 30%, while the calculated residual ethanol values ranged from 70% to 99% (Table 1). We then generated scatter plots to compare the storage time and the values of calculated lost ethanol and calculated residual ethanol (Figure 1). There was no correlation between the calculated lost ethanol and the second ethanol analysis day ($p = 0.831$). Similarly there was no correlation between the calculated residual ethanol and the second ethanol analysis day ($p = 0.831$).

Table 1. Ethanol concentrations and calculated values for study A.

Variables	Mean±SD	Minimum-Maximum value
Dwelling times of the sera on -80 ºC (day)	126.7 ± 66.22	1-295
First Ethanol Concentrations (mg/dL)	164.48±79.89	28.5-395.2
Second Ethanol Concentrations (mg/dL)	139.67±67.31	25.7-354.7
Calculated Lost Ethanol (%)	14.70±5.99	1-30
Calculated Residual Ethanol (%)	85.30±5.99	70-99

SD, Standard deviation

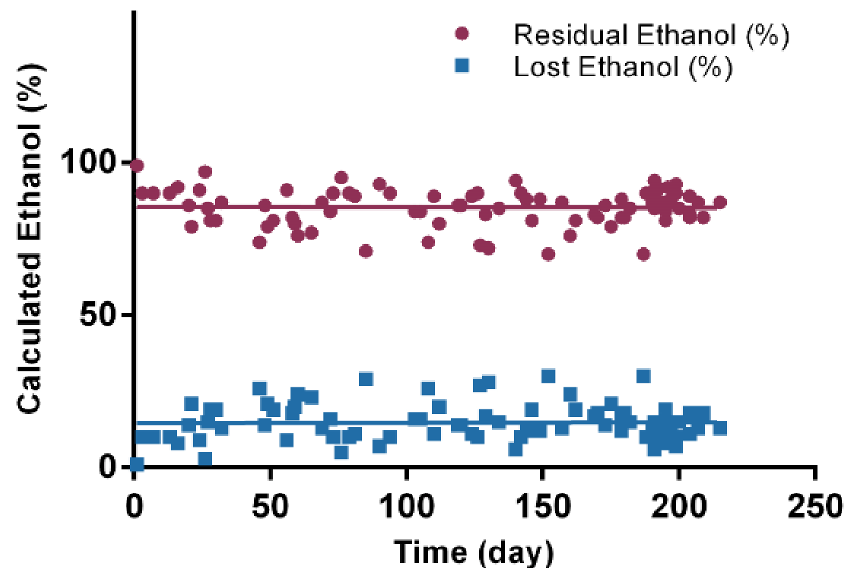

Figure 1. Scatter plots comparing dwelling time and values of calculated lost ethanol (%) and calculated residual ethanol (%).

Results of Study B

For study B, we analyzed 34 samples for which ethanol concentration had been measured within 180 days of the working day. Ethanol concentrations from both analyses were compared. The first ethanol concentration ranged from 27.3 to 270.40 mg/dL (153.05 ± 68.550), while the second ethanol concentration ranged from 23.5 to 256.10 mg/dL (133.39 ± 60.50). A significant difference was detected between the results of the two analyses ($p < 0.01$).

Inter-assay coefficient variations (CVs) were calculated from July 2015 to February 2016. Data are shown in Table 2.

Table 2. Inter-assay coefficient variations.

Interval	CV (%)[a]	CV (%)[b]
July 2015	4.00	2.51
August 2015	3.93	2.53
September 2015	4.15	2.27
October 2015	4.99	3.57
November 2015	3.03	2.48
December 2015	3.53	2.44
January 2016	3.94	4.46
February 2016	3.95	2.65
Mean CV	3.94	2.86

CV, coefficient variations; a at concentration of 48.4 mg/dL; b at concentration of 148.8 mg/dL.

Discussion

In most countries, alcohol consumption is a common and legal social activity (14). Alcohol-impaired driving accounts for approximately 30% of all traffic-related deaths (5). For this reason, accurate measurement of blood–ethanol concentration is very important with respect to forensic evidence, to inform high levels of conclusiveness in forensic and laboratory medicine and in related lawsuits (15).

In the current study, of chief concern in analyzing blood–ethanol concentrations was the effect of storage conditions. We investigated the stability of blood–ethanol concentration within an eight-month storage period, by time and whether there were any differences between blood–ethanol concentrations before and after six months of storage. We established that blood samples analyzed for ethanol content remained stable for quite some time (Figure 1), as long as the blood samples had not been exposed to vaporization (2). Unlike this stable course of blood–ethanol concentration, we found a significant difference between concentration levels before and after six months of storage: differences were as observable as they were apparent. However, it has been suggested that vaporization, chemical reactions, or the diffusion of ethanol from polypropylene tubes may reduce blood–ethanol concentration, even some time later (2, 13, 16). To the best of our knowledge, this study is the first to investigate the stability of blood–ethanol concentrations in samples stored at –80°C for six months.

In recent years, besides enzymatic ADH, a large number of new and reliable ethanol determinations have been developed, such as headspace gas

chromatography, headspace solid-phase microextraction, capillary gas chromatography, and proton nuclear magnetic resonance spectroscopy (15, 17-19). Reliability of ethanol measurement is contingent on the control of the pre-analytical phase-the testing phase in which approximately nine-tenths of all diagnostic errors occur. It is also where the reliability of sample analysis-especially with stored samples-is called into question (9).

It has been emphasized that blood samples analyzed for the presence of ethanol should be retained for long time intervals, given the possibility that criminal prosecution might occur at some later date (6). To ensure the test result reliability of biological samples thawed several months later, these samples should be stored in appropriate conditions. To date, several studies have described the stability of ethanol in biological samples (10-13, 20, 21).

Kocak et al. (13) established that storing samples at –20°C is suitable for a three to four-month period, but that it is not acceptable for a five-month period. This difference has been attributed to the escape of ethanol vapor from tubes and chemical reactions with ethanol resulting from the presence of air above the sample. In our study, storage duration did not appear to be an important factor to affect blood–ethanol concentration.

In addition to storage conditions, blood sample type has also been investigated with regards to its effect on determinations of blood–ethanol concentration. Penetar (12) proposed that plasma and serum ethanol concentrations are generally and approximately 11% higher than whole blood–ethanol concentrations, across all time points (up to 180 minutes after alcohol consumption), across all collection tubes (e.g., gray, lavender, red-capped tubes), and across a number of processing conditions (i.e.., analysis as soon as possible, after storage at room temperature, and after storage at 4°C). Additionally, Barnhill (20) established that the serum/whole blood–ethanol ratio ranged from approximately 1.12 to 1.18, depending on serum–ethanol concentration. Therefore, Barnhill has precluded the employability of the general serum/whole blood–ethanol ratio. Similar to Barnhill's serum/whole blood–ethanol ratio range of 1.12–1.18, Charlebois (21) et al. asserts that the serum/whole blood–ethanol ratio ranges from almost 1.04 to 1.26, with a mean ratio of 1.14.
Jones (22) investigated the effect of the baseline ethanol level and storage interval on the results of a second ethanol determination. That study found that reduced ethanol levels correlate positively with the length of storage and the original blood–ethanol concentration level. Additionally, it has been proposed that the more concentrated the first ethanol determination result is, the more likely we are to reach a "true result" with a second determination (22). Unlike the results of that study, the current study found that the concentration at the first ethanol determination has no influence on the result of the second determination.

There is some consensus among the results of stability studies that have investigated the storage conditions of blood samples analyzed for ethanol content; this consensus is within the bounds of desirable imprecision of RiliBÄK (<9%) (2) or the allowable total error according to CLIA'88 (<25%) (13). In our study, inter-assay CVs calculated from July 2015 to February 2016 did not exceed the desirable imprecision of RiliBÄK; however, deviations from the results of the first analysis exceeded the allowable total error according to CLIA'88. The observed deviations

were 1–30%; this discrepancy may have resulted from delays in freezing blood samples analyzed for ethanol content. Unfortunately, given the retrospective nature of our study, this experimental component could not be controlled for.

This study has some limitations. Determinations of blood–ethanol concentration were not performed through the use of more reliable methods, such as gas chromatography. Limitations inherent in the enzymatic ADH method could have affected the first or second set of results. Secondly, as mentioned, this study has a retrospective design; had this study instead a prospective schedule, all samples would have consistently undergone centrifugation, analysis, and proper storage as soon after collection as possible.

Conclusion

We conclude that blood samples analyzed for ethanol content may be stored at –80°C for many months, provided that analysis is undertaken and the post-analysis freezing of blood samples occurs as promptly as possible. In many countries, there has been a need to establish rules concerning storage conditions and a "storage life" for blood samples analyzed for ethanol content. Our study may help satisfy the need to develop more appropriate standard practices for forensic science purposes.

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