Ethanol concentration changes in blood samples during medium-term refrigerated storage

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Abstract. – OBJECTIVE: Stability of blood alcohol concentration (BAC) in laboratory samples is of great importance when it is necessary to perform repeated analyses.

MATERIALS AND METHODS: We have analyzed the stability of BAC in 50 samples, which were taken from apprehended drivers, kept at -18°C, without preserving agents. Quantitative analyses were performed using headspace sampling gas chromatography (HS-GC) with flame ionizing detection (FID). Samples were analyzed immediately after collection (C_1), and after 60 (C_{60}), 120 (C_{120}) and 180 (C_{180}) days. A group of 50 samples, which were kept closed for 180 days at -18°C, was utilized as a control.

RESULTS: We found a significant decrease in BAC between C_1 and C_{180} (= 0.224; SD= 0.144; t = 10.98; $p < 0.001$), and between C_1 and C_{60}, C_{60} and C_{120}, C_{120} and C_{180}. There was a significant positive correlation (r=0.8) between starting concentration C_1, and the value of BAC changes (ΔC). Linear regression analysis (R^2=0.64) implies the degree of validity to the proposed model of ΔC change regarding initial BAC. There were significant changes in ΔC between the two groups.

CONCLUSIONS: These data underline the significance of air chamber percent (CA%) and ethanol evaporation due to ventilation between liquid and gas phase as a mechanism of ethanol decay.

Key Words:
Ethanol, Blood, Analysis, Stability, Headspace gas chromatography.

Introduction

Alcohol is widely used and most commonly analyzed toxic substance in forensic toxicology laboratories[1]. The contribution of driving under the influence of drugs to road crashes is non-ne-gligible, while that of alcohol is much greater than that of any other drugs[2]. In Europe 90.5% of injured drivers and 87% killed drivers had a blood alcohol concentration (BAC) of ≥ 0.5 g/L, which is the acceptable limit of blood alcohol content before a person is charged with a crime in Italy. The median value for alcohol concentration in injured drivers was 1.6 g/L, while in killed drivers was 1.7 mg/dL, respectively[3]. The recent introduction of law No. 41/2016 in Italy represents a significant step forward, especially regarding the punishment increase for offenses committed by people driving under the influence of alcohol or psychotropic drugs (paragraph No 2, section 589-bis P.C.)[4].

One of the major concerns in performing toxicology analysis of alcohol is its stability in forensic specimens. The concentration can rise during the time of storage, or it may be susceptible to losses due to evaporation, microbial degradation and oxidation[5]. This is important when performing repeated analyses, for example as a part of quality control and assurance, or if analyses are requested by the court to verify the initial results[6,7]. When presenting and interpreting results for the court, the ability to produce reliable results and interpret any significant changes is crucial in establishing the credibility of data. Many authors imply the significance of pre-analytical factors, and studies have shown that BAC decreases during storage, depending on the time and temperature of preservation, whether preservatives (e.g., sodium fluoride) have been added, integrity of vacutainers and seals and percent of air chamber (CA%) within the vessel[8-10]. These aspects are of great importance, especially in developing countries where procedures of sampling, collection and conservation of specimens are frequently

deficient, along with the lack of proper quality control. Some authors point to the significance of method imprecision[11]. The concept of evaluating differences in laboratory results has long been discussed in laboratory medicine[12].

This study analyzes the stability of alcohol in forensic blood specimens obtained from living persons, stored for 6 months in a refrigerated state at -18ºC, which were intermittently opened two times during the storage, mimicking possible quality control sampling and/or court orders for repeated analyses. Changes in concentration were evaluated as a function of initial BAC and the time of storage.

Materials and Methods

Materials

Blood samples were obtained from apprehended drivers and sent to Clinical Center of Vojvodina, Center for Forensic Medicine, Toxicology and Molecular Genetic, Novi Sad, Serbia, for determination of alcohol levels. We have analyzed 50 blood samples, taken both from men and women, with no regards to their age. Lower limit for inclusion was set to 0.3 mg/ml, which is statutory blood alcohol limit for driving in Serbia, and the upper limit was set at 3.5 mg/ml, because there were so few instances where BAC levels exceeded this limit[13].

Methods

Blood specimens were taken from the antecubital vein, into two tubes of 6 ml with lithium heparin (BD Vacutainer®, BD-Plymouth, UK) after the skin was cleansed with soap and water. The evacuated tubes were made of glass, with a gray rubber stopper and 102 I.U. of lithium heparin as the anticoagulant. Immediately after evacuating, blood tubes were inverted several times to uniformly mix heparin. The air chamber percent (CA%) was set to 20%. One tube stayed closed at the refrigerator, at +4ºC and was analyzed in the first 24h after the reception. The second tube remained closed and refrigerated at -18ºC, for the duration of 180 days. After 60 days tubes were defrosted, stayed at +4ºC for 24h, and then for a few minutes at room temperature aliquots were removed for analyses. After analysis tubes were again refrigerated at -18ºC. The same procedure was repeated after 120 days, and finally after 180 days. The control group was represented by 50 blood specimens that, after initial analysis, remained unopened for 180 days, and then were reanalyzed for BAC, using the same, afore mentioned procedure.

The concentration of alcohol was determined by headspace gas chromatography with flame-ionizing detection. The routine procedure was described in detail[14]. GC equipment used was headspace sampler Agilent G1888 and GC Agilent G6850, and software used for GC data analysis was GC/MSD ChemStation, running on Windows XP. The columns used were DB-ALC1 30-m columns. The samples were incubated for 12 min at 55ºC before analysis. The GC was calibrated before every run and controls were placed every 5th place to retain reliability and consistency of results. Aliquots of the whole blood, 100 µl, were diluted with 1:10 aqueous n-propanol as the internal standard, in 20 ml glass vials, which were immediately sealed with rubber stoppers and crimped with aluminum caps. All determinations of BAC were done in duplicate by two technicians. Limit of detection (LOD) was determined at 0.0025 and limit od quantitation (LOQ) at 0.01 mg/ml.

Statistical Analysis

Statistical analysis was performed in MS Office Excel 2016, using Analysis Tool Pak. Conventional statistical parameters were determined first. The significance of the difference between mean values of analyses in the first, second, fourth and sixth month was determined using t-test. The correlation test was used to determine the correlation coefficient between initial BAC and change in BAC (ΔC) after the sixth month, which was also analyzed using linear regression analysis. As a limit of statistical significance value p 0.05 was used.

Results

In all of 50 samples, there was a significant decrease in BAC between first and sixth month (C_1 and C_{180}), with a mean value () of decrease 0.224 mg/ml, and SD 0.144 mg/ml, the coefficient of variation (CV) being 0.64. All values exceeded LOD, and LOQ, the lowest change being 0.029 mg/ml and highest 0.643 mg/ml. The decrease in BAC between C_1 and C_{180} was statistically significant with t=10.98 (df=49; p<0.001) (Tables I, II).

A significant decrease in BAC was also found when groups of intermittently opened and analyzed samples were compared (BAC C_1 – BAC C_{60}; BAC C_{60} – BAC C_{120} and BAC C_{120} – BAC C_{180}),

Table I. Descriptive statistical parameters for initial analysis of samples (C1), analysis after 60, 120 and 180 days (C_{60}, C_{120}, C_{180}), and changes in BAC between C1 and C_{180}

C_1		C_{60}		C_{120}		C_{180}		ΔC	
$\bar{x}$	**1.565**	$\bar{x}$	**1.471**	$\bar{x}$	**1.404**	$\bar{x}$	**1.341**	$\bar{x}$	**0.224**
SD	0.819	SD	0.763	SD	0.74	SD	0.709	SD	0.144
Var	0.672	Var	0.582	Var	0.547	Var	0.50	Var	0.021
Range	3.074	Range	2.757	Range	2.844	Range	2.725	Range	0.643
Min	0.314	Min	0.313	Min	0.303	Min	0.285	Min	0.029
Max	3.388	Max	3.07	Max	3.147	Max	3.01	Max	0.672
Count	50	Count	50	Count	50	Count	50	Count	50

with t values of 6.386; 5.898 and 7.565 respectively (df=49; *p*<0.001) (Tables III-V).

In all tests one tail distribution was used, because neither there were cases of increase in BAC, nor it was expected to be, so only changes in decrease were analyzed. There is a strong positive correlation between initial BAC and a decrease in BAC (ΔC), with r=0.8, which can be seen on a scattered plot.

Linear regression model was used to estimate ΔC when initial BAC is known. The coefficient of determination (R^2) was 0.640; F=85.04; *p*<0.001, which implies the degree of proposed model validity when initial BAC is known (Figure I).

When comparing changes in BAC with the control group of unopened samples, there was statistically significant difference, with a much higher decrease in BAC in opened samples. The mean decrease in BAC in the opened tubes was 0.224±0.144 compared to 0.117±0.084 mg/ml in unopened ones (*t*=4.53; *p*<0.001).

Discussion

It is important to investigate the stability of alcohol in biological samples[15]. This is very important if the results of analysis are close to a statutory alcohol limit for driving, or when there
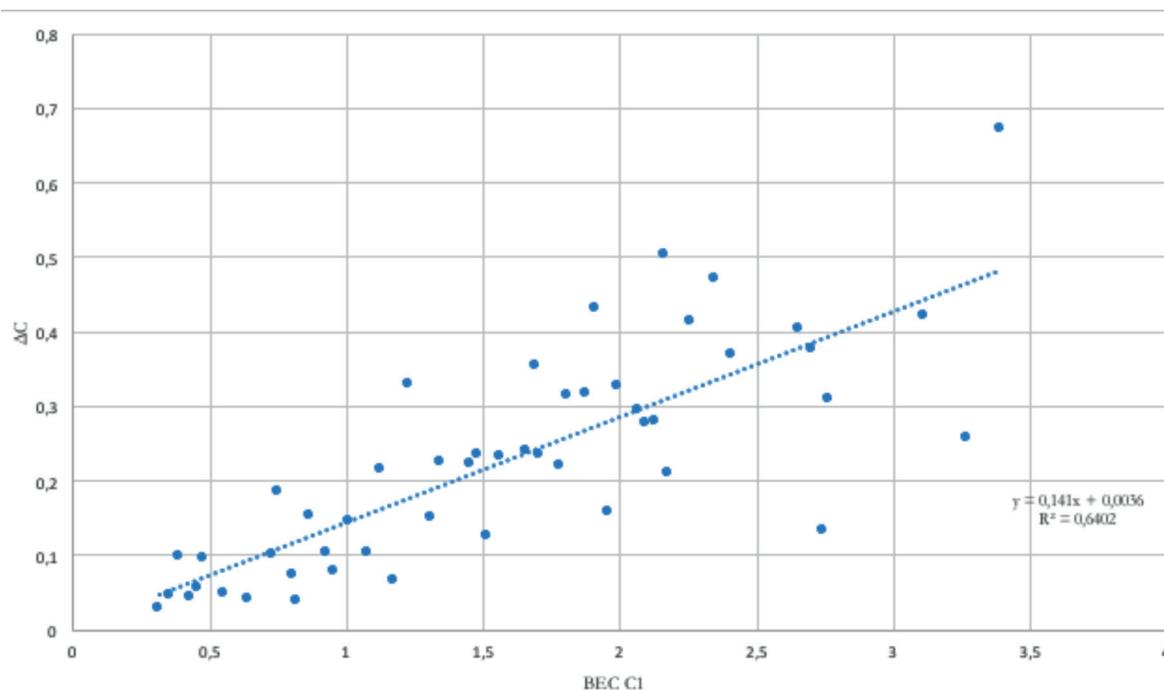

Figure 1. Relationship between decreases in blood-ethanol concentration (BEC) during storage and the starting BEC.

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Table II. *t*-test for two paired samples, between initial analysis and after 180 days

	C1	C180
$\bar{x}$	1.565	1.341
Variance	0.672	0.503
Observations	50	50
df	49	
t Stat	10.981	
p (T≤*t*) one-tail	4.107e-15	
t Critical one-tail	1.676	

Table III. *t*-test for two paired samples, between initial analysis and after 60 days

	C1	C60
$\bar{x}$	1.565	1.471
Variance	0.672	0.582
Observations	50	50
df	49	
t Stat	6.386	
p (T≤*t*) one-tail	2.957e-08	
t Critical one-tail	1.676	

is a court request to verify the validity of the first analysis[16]. Because injured and killed drivers were in large percent under the influence of elevated alcohol levels, new and improved laws may serve as an example regarding the punishment increase for offenses committed by people driving under the influence of alcohol[4]. Several mechanisms of alcohol decrease in biological samples were proposed. Since ethanol is a volatile substance, BAC may be expected to decrease during the time. BAC in samples may decrease due to a leakage in the container or by biochemical degradation if a preservative, e.g., sodium fluoride had been omitted[17]. Contamination of specimen by yeasts or bacteria from the skin at the sampling site or the equipment used to handle the blood may lead to production or loss of ethanol by the action of microbes[18]. Some authors stated that the use of preserving agents, such as sodium fluoride in human blood samples, does not improve the results, especially if the samples were taken with a sterile syringe and kept at low temperatures. Under these conditions, human blood samples can be analyzed after 2 weeks with no substantial variation regarding the blood alcohol levels obtained the first day[19]. Other studies[20], based on analysis of whole blood and serum samples, which were kept for several weeks at different temperatures, indicated that

samples maintained at higher temperatures showed significant decay only after a month, especially in whole blood samples. In a separate study, Jones et al[21], pointed to the significance of the imprecision of an analytical method. It is important to take it into account when changes in the concentration of an analyte as a BAC during storage were to be considered. Since imprecision tends to change as a function of concentration, the critical differences need to be calculated at different levels of BAC. In this study, it was concluded that decrease in BAC was to be analytically significant if they exceeded 0.013 (2.6%), 0.028 (1.9%) and 0.045 mg/g (1.8%), respectively at starting concentrations of 0.5, 1.5 and 2.5 mg/g[17]. Decreases in BAC during storage also depend on the length of storage. Because of a large residual standard deviation and coefficient of variation, it is not advisable to make an estimate of the loss of ethanol during storage by use of the regression equation, nor to calculate initial BAC. In a study dealing with the kinetics of ethanol degradation as a function of time, chamber air percent (CA%), temperature and initial concentration, a group of authors came with a formula that intends to predict, with minimal error, the initial ethanol concentration in samples stored at a temperature and constant air chamber percentage. If the time of the sample kept under these conditions

Table IV. *t*-test for two paired samples, between analysis after 60 and 120 days.

	C60	C120
$\bar{x}$	1.471	1.404
Variance	0.582	0.547
Observations	50	50
df	49	
t Stat	5.898	
p (T≤*t*) one-tail	1.675e-07	
t Critical one-tail	1.676	

Table V. *t*-test ffor two paired samples, between analysis after 120 and 180 days.

	C120	C180
$\bar{x}$	1.404	1.341
Variance	0.547	0.503
Observations	50	50
df	49	
t Stat	7.565	
p (T≤*t*) one-tail	4.438e-10	
t Critical one-tail	1.676	

is known, by measuring the ethanol concentration at a specific moment it is possible to estimate the alcohol concentration at zero time and thus predict blood alcohol levels[12].

We have also found that during storage, there was a significant decrease in BAC. In the present study, no preservative was used, but the significance of it was considered unnecessary, as previously mentioned, since all pre-analytical factors of analysis were fulfilled, and samples were kept at very low temperatures. Other authors also found that repeated opening of blood tubes to remove aliquots for analysis resulted in a greater loss of ethanol, which suggests that ventilation with ambient air plays a role in the degradation process[10]. However, it seems to disagree on a significance of different mechanisms of ethanol decay. Jones stated that losses of alcohol from the air-space when aliquots of blood are removed for analysis is not a plausible explanation because at room temperature the blood/air partition coefficient of ethanol is approximately 5000:1. The amount of alcohol that enters the air-phase above the blood at room temperature is negligible compared to the total amount of ethanol in the liquid phase[22,23]. When dealing with kinetics of ethanol degradation, other authors stated that the percent of air in the chamber (CA%) is of great importance, because the greater the CA%, the greater is the decrease in BAC during time[9], which cannot be explained in another manner than loosing alcohol due to ventilation between liquid and gas phase. Other than ventilation, there are also mechanisms of alcohol decay that involve a non-enzymatic oxidation reaction by oxyhemoglobin within the erythrocytes, but this process is not dependent on CA%[8].

Conclusions

During the storage of blood specimens at -18°C, with CA% of 20% and without the use of fluoride as preserving agent, there was a significant decrease in BAC, and significantly more so in a group of samples that were repeatedly opened two times during the storage, which implies the significance of CA% and ethanol evaporation due to ventilation between liquid and gas phase as a mechanism of alcohol decay. A number of alcohol losses are in a positive correlation with initial BAC and days of storage. Not one sample showed an increase in ethanol concentration, which asser-

ts the statement that it is not necessary to add preservative when pre-analytical factors are fulfilled, and samples kept at very low temperatures.

Conflicts of interest
The authors declare no conflicts of interest.

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