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Research Article
Interpretation of Ethanol Concentration Changes in Postmortem Blood and Vitreous Samples During Six Months of Refrigerated Storage

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Abstract

Background and Objective: Blood alcohol concentration (BAC) may rise during storage, or it may decrease. Femoral vein is the best place for taking postmortem blood samples. Urine and vitreous humour (VH) should be obtained. The aims of the study was to determine if there were significant changes in BAC and VH during storage; in which sample is less susceptible to changes and if could VH be used to estimate BAC in postmortem specimens. **Materials and Methods:** Samples obtained during autopsy of 60 deceased persons were kept at -80°C for six months. Headspace Gas Chromatography with Flame Ionization Detection (HS-GC-FID) was used for quantitative analyses. Samples were analysed immediately (C_1) and after 180 days (C_{180}). The level of marginal statistical significance of $p<0.05$ was used. **Results:** There was a significant decrease in BAC between C_1 and C_{180} ($\bar{\chi} = 0.139$, SD = 0.097, t = 11.09 and $p<0.001$), as well as in VH ($\bar{\chi} = 0.07$, SD = 0.063, t = 8.61 and $p<0.001$). Mean changes in BAC and VH show significant differences (t = 4.635 and $p<0.001$). Calculated VH/BAC ratio in initial analysis ($\bar{\chi} = 1.161$) and after 180 days ($\bar{\chi} = 1.244$) show a significant difference (t =-2.296 and $p<0.05$). There is a high linear correlation between VH and BAC values ($y = 0.8622x+0.0099$, $R^2 = 0.9537$, $y = 0.7948x+0.0323$ and $R^2 = 0.9427$). **Conclusion:** The BAC and VH ethanol levels significantly decreased during six months. Mean VH/BAC ratio corresponds to previous studies, as well as linear regression equations which propose calculating BAC when VH is known. Individual variations in VH/BAC ratio suggest that it is not recommended to use any of these values to predict the other one.

Key words: Ethanol, blood alcohol concentration, vitreous humour, headspace gas chromatography, toxicology

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The use of alcoholic beverages is a major factor in fatal accidents, suicides and violent crimes and high blood alcohol concentration (BAC) levels are often found in cases of deaths due to natural causes as well[1,2]. Alcohol is the most common toxic substance analyzed in forensic and clinical toxicology laboratories[2,3]. Analyzing and interpreting BAC in autopsy specimens is a major part of forensic toxicology workload[3]. Quantitative analysis of postmortem ethanol is a relatively simple procedure where accurate, specific and precise results can be obtained[4,5]. Interpretation of postmortem BAC, where one is to assume antemortem levels at the time of death is a different problem, with many difficulties[6-9].

Stability of ethanol in postmortem samples is another main question. During storage BAC can rise or it may decrease due to different mechanisms, such as evaporation, oxidation and microbial degradation[10-13]. This is of major importance if repeated analyses are requested by the court, but also as a part of internal and/or external quality control procedures[14,15]. If the obtained data are to be credible, then producing reliable results and scientific interpretation of any significant change is crucial.

The important factors to consider in these analyses are usually related to the condition of the body, which depends on the time of death, various environmental factors and the nature of the specimen collected, etc. Microbial activity, mainly by glucose fermentation, can lead to a rise in BAC, but this can also be the result of postmortem ethanol diffusion, from the stomach to the bloodstream[16,17]. Biological specimens must not be contaminated by ethanol or any other solvent during medical procedures.

Contrary to the previous, BAC can decrease during storage, which depends on the presence of preservatives (sodium fluoride), time and temperature of preservation. The integrity of vacutainers and rubber seals and especially the percent of air in a vessel (CA%) are also factors that can influence postmortem BAC while preserving specimens. Pre-analytical factors are also of significance[15,18,19].

Guidelines for collecting postmortem toxicology specimens suggest that the femoral vein is the best place for taking postmortem blood samples. If possible, urine and vitreous humor (VH) should also be obtained[20-22].

All these aspects are of great importance, especially regarding the analysis and interpretation of data in developing countries, where there is usually a lack of appropriate quality control and assurance regarding specimen sampling, collection and conservation.

The main aims of this study were to determine if there were significant changes in BAC and VH concentrations during 6 months of refrigerated storage and also determine the use of VH concentration in certain cases viable method to estimate BAC levels.

MATERIALS AND METHODS

Study area: This research was conducted from January to December, 2022 at The Centre for Forensic Medicine, Toxicology and Molecular Genetics, Clinical Centre of Vojvodina, Novi Sad, Vojvodina, Serbia.

Blood and vitreous samples were obtained from autopsy specimens of deceased persons who were sent to The Centre for Forensic Medicine, Toxicology and Molecular Genetics in Novi Sad in order to perform an autopsy and determine ethanol levels. While 60 blood and vitreous samples were analyzed, with no regard to age or sex. Present study results as well as previous studies implied that the lower level of inclusion was set at 0.1 mg/mL and the upper one at 3.5 mg/mL[23].

Blood samples from the femoral vein were taken before autopsy, into 6 mL tubes filled with lithium heparin (BD Vacutainer®, BD-Plymouth, United Kingdom). Sterile needles and syringes were used. Vitreous specimens were also obtained during autopsy, using small sterile needles and syringes, after penetrating the ocular bulbs in the lateral eye angle. Glass evacuation tubes with gray rubber stopper and 102 I.U. of anticoagulant-lithium heparin was inverted a few times in order to mix heparin with biological sample. Usual percent of air (CA%) was set at 20% for blood and 50% for vitreous because of small quantities. Both specimens were closed and then put in the refrigerator, at 4°C. These samples were analyzed within the first 24 hrs after the reception. After initial analyses the tubes with blood and vitreous specimens were frozen at -80°C and remained in these conditions for 180 days. After 6 months the specimens were unfrozen for 24 hrs at 4°C and then analyzed again. Headspace Gas Chromatography with Flame Ionization Detection (HS-GC-FID) was used to determine the concentration of alcohol. The procedures of preparing biological samples and ethanol analysis were modified with respect to the methods reported in the literature[24,25].

To biological samples (0.1 mL) in a 20 mL HS vial, a solution of the internal standard (IS) of tert-butanol (0.1 mL) and sodium chloride (0.5 g) were added. Analyses were performed using Agilent 8860 GC System with Agilent 7697 A Headspace sampler, Agilent Technologies, Santa Clara, United States, as described in detail by

Djurendic-Brenesel *et al.*[25]. The retention times for ethanol and IS were 1.03 and 1.29 min, respectively. Working solutions of ethanol (80) and IS (0.8 mg/mL) were prepared by diluting the original standard solutions (in deionized water) purchased from Sigma-Aldrich (Steinheim, Germany). Calibration samples were prepared by adding a working solution of ethanol to a 1 mL free human blood and vitreous humor, to achieve the concentration range: 0.2-3.2 mg/mL. Aliquots of calibration samples (0.1 mL) and 0.1 mL of working solution of IS were placed in 20 mL HS vials, to which 0.5 g sodium chloride had been previously added. Every sample was analyzed three times, by two laboratory technicians and the mean values were used for further calculations. The ratio of the peak areas of ethanol and that of IS were presented as a function of the substance concentration using linear regression method. The calibration curves were linear with a correlation coefficient of 0.999. The limit of detection (LOD) at 0.0025 mg/mL, the limit of quantitation (LOQ) at 0.01 mg/mL was established and the recovery of ethanol in the range 97-99%. The reported method was validated in accordance with the International Guidelines for Bioanalytical Methods and the guidelines for laboratories for the analysis of substances of abuse for toxicological-forensic and medico-legal purposes[26-28].

Ethical consideration

Ethical approval: The research was approved by the Ethical Committee of the Clinical Centre of Vojvodina.

Statement of human and animal rights: The authors state that this research was conducted in accordance with the Helsinki Declaration as revised in 2008.

Statement of informed consent: The informed consent approval was not necessary to obtain since all biological samples were taken from deceased persons who were sent by court order to perform an autopsy and all samples taken were routinely collected as a part of the autopsy procedure.

Statistical analysis: Microsoft Office Excel 2021 used to perform statistical analyses, both with conventional tools and using Data Analysis ToolPak. Along the conventional statistical parameters, which were determined first, appropriate t-tests were used to determine statistical significance, while setting the limit at $p<0.05$. Linear regression analyses were used to determine the causative relationship between BAC and VH in the first and the sixth month, as well as the relationship between initial concentrations of BAC and VH and changes (ΔC) after six months.

RESULTS

Blood alcohol concentration (BAC): The mean value ($\bar{\chi}$) of ethanol concentration decrease in blood samples (ΔCb) was 0.139 mg/mL, with SD being 0.097 and the range 0.419 mg/mL, minimum being 0.007 mg/mL and maximum 0.426 mg/mL. A significant decrease found in BAC between the first (C_1) and the sixth month (C_{180}), with $t = 11.09$ ($p<0.001$) (Table 1 and 2).

Vitreous humour (VH) concentrations: The mean value ($\bar{\chi}$) of ethanol concentration decrease in VH (ΔCv) was 0.07 mg/mL, with SD being 0.063 and the range 0.285 mg/mL, minimum being 0.004 mg/mL and maximum 0.285 mg/mL. It is found that there was a significant decrease in ethanol concentration between the first (C_1) and the sixth month (C_{180}), with $t = 8.61$ ($p<0.001$) (Table 3 and 4).

There were no cases of increase in ethanol concentrations, so only changes in decrease were analysed, hence the use of one tail distribution.

BAC and VH relation: Using t-test for two samples assuming unequal variance we found that there was a statistically significant difference between mean changes in ethanol concentration in blood (ΔCb) and vitreous samples (ΔCv), $t_{stat} = 4.635$ ($p<0.001$) (Table 5).

In order to establish the relationship between initial BAC and VH levels and their changes after 180 days (ΔCb and ΔCv) a linear regression equation was calculated, using general formula $y = mx+b$, where x equals BAC and VH ethanol concentrations and y equals ΔCb and ΔCv. The results were shown in linear regression plots in Fig. 1 and 2.

The regression analysis for BAC yielded the equation:

$$y = 0.1113x-0.0129 \text{ (mg/mL)}$$

where, y corresponds to ΔCb and x to BAC. The coefficient of determination (R^2) was calculated at 0.5469 (Fig. 1), $F = 1.487e^{-11}$.

The regression analysis for VH yielded the equation:

$$y = 0.0384x+0.0095 \text{ (mg/mL)}$$

where, y corresponds to ΔCv and x to VH concentrations. The coefficient of determination (R^2) was calculated at 0.1996 (Fig. 2), $F = 0.0003$.

These results showed that although there is a positive relation between initial concentrations in blood and vitreous and their changes after 180 days ($F = 1.487e^{-11}$ for BAC and

Table 1: Descriptive statistical parameters for ethanol concentrations in postmortem blood samples

Value	C_1 (mg/mL)	C_{180} (mg/mL)	ΔCb (mg/mL)
Mean ($\bar{\chi}$)	1.371	1.231	0.139
SD	0.648	0.58	0.097
Range	2.765	2.46	0.419
Minimum	0.389	0.312	0.007
Maximum	3.154	2.772	0.426

Table 2: t-test for two paired samples, mean values of initial analysis (C_1) and after 180 days (C_{180}), blood samples

Value	C_1 (mg/mL)	C_{180} (mg/mL)
Mean	1.371	1.231
Variance	0.420	0.336
Observations	60	60
Df	59	
t-Stat	11.09	
p (T≤t) one tail	$2.306e^{-16}$	
t critical one-tail	1.671	

Table 3: Descriptive statistical parameters for ethanol concentrations in postmortem vitreous samples

Value	C_1 (mg/mL)	C_{180} (mg/mL)	ΔCv (mg/mL)
Mean	1.579	1.508	0.07
SD	0.734	0.708	0.063
Range	3.186	2.945	0.281
Minimum	0.386	0.377	0.004
Maximum	3.572	3.322	0.285

Table 4: t-test for two paired samples, mean values of initial analysis (C_1) and after 180 days (C_{180}), vitreous samples

Value	C_1 (mg/mL)	C_{180} (mg/mL)
Mean	1.579	1.508
Variance	0.539	0.502
Observations	60	60
Df	59	
t-Stat	8.61	
p (T≤t) one tail	$2.56e^{-12}$	
t critical one-tail	1.671	

Table 5: t-test for two samples assuming unequal variance, comparing ΔCb and ΔCv

Value	ΔCb (mg/mL)	ΔCv (mg/mL)
Mean	0.139	0.07
Variance	0.009	0.003
Observations	60	60
Df	101	
t-Stat	4.635	
p (T≤t) one tail	$5.33e^{-6}$	
t critical one-tail	1.66	
p (T≤t) two tail	$1.067e^{-5}$	
t critical two-tail	1.983	

Table 6: VH/BAC ratio in initial samples and after 180 days

Values	$\Delta Cv/\Delta Cb_1$	$\Delta Cv/\Delta Cb_{180}$
Mean ($\bar{\chi}$)	1.161	1.244
SD	0.17	0.223
Range	0.97	1.369
Minimum	0.71	0.758
Maximum	1.68	2.127

F = 0.0003 for VH) there is no linear model which can be applied and that initial levels are just one of the factors which influence the changes in BAC and VH concentrations during storage.

When comparing the relations between VH and BAC (ΔCv/ΔCb) it has found that in the first analyses $\bar{\chi}$ = 1.161, SD = 0.17 and in the 6th month $\bar{\chi}$ = 1.244, SD = 0.223. This means that 95% of the same population falls

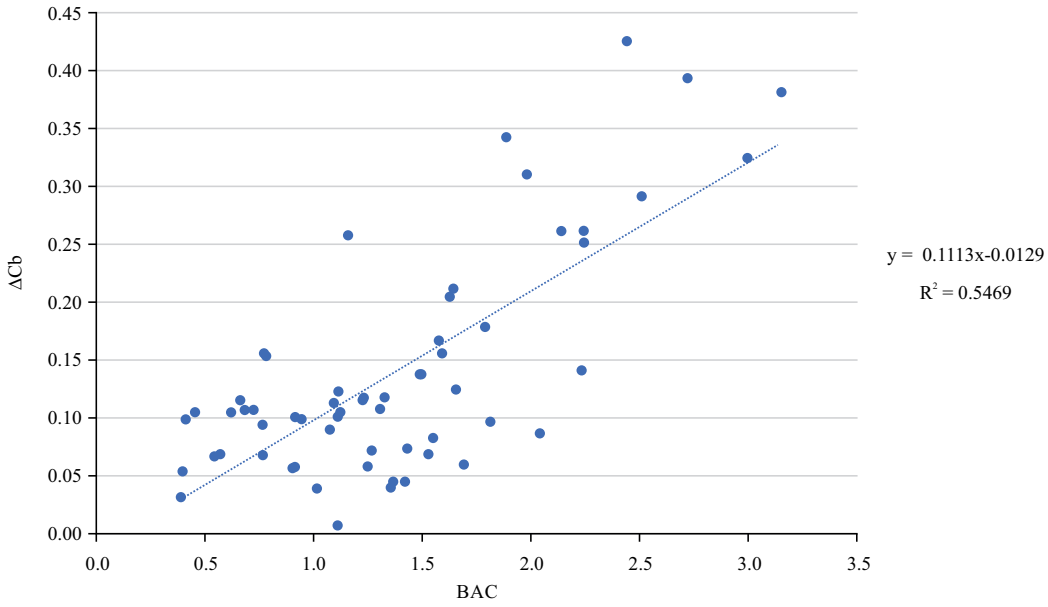

Fig. 1: Linear regression plot for BAC (x) and ΔCb (y)

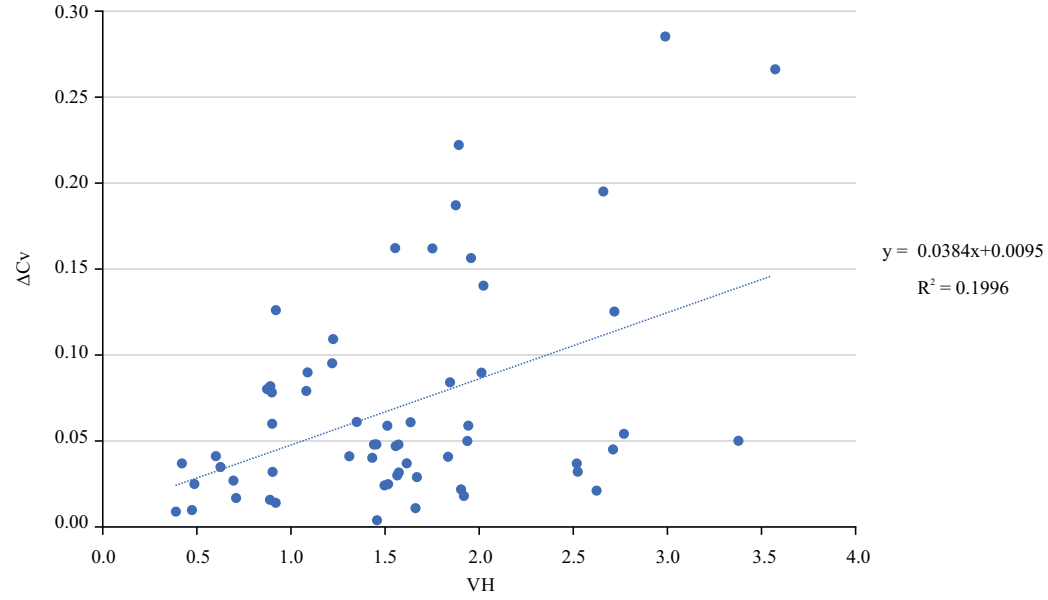

Fig. 2: Linear regression plot for VH (x) and ΔCv (y)

somewhere in between VH/BAC ratio of 0.828 and 1.494 for initial analysis and 0.807 to 1.681 after six months ($\bar{\chi} \pm 1.96 \times SD$) (Table 6).

In order to establish the relationship between BAC and VH a correlation coefficient and regression equation were calculated. Data analysis was performed by linear regression (y = mx+b) and Fig. 3 and 4 show the linear plot between ethanol concentrations in VH (x) versus BAC (y).

The regression analysis in the initial moment yielded the equation:

$$y = 0.8622x+0.0099 \text{ (mg/mL)}$$

where, y corresponds to BAC and x to VH ethanol levels. The coefficient of determination (R^2) was calculated at 0.9537 (Fig. 3) $F = 2.118e^{-40}$.

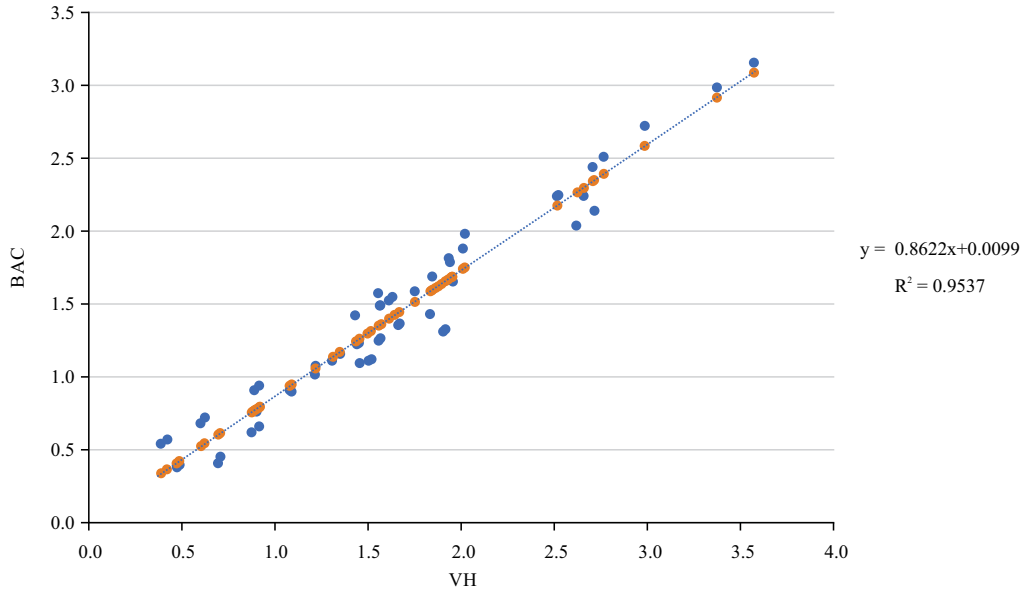

Fig. 3: Linear regression plot between VH (x) and BAC (y) in initial analysis

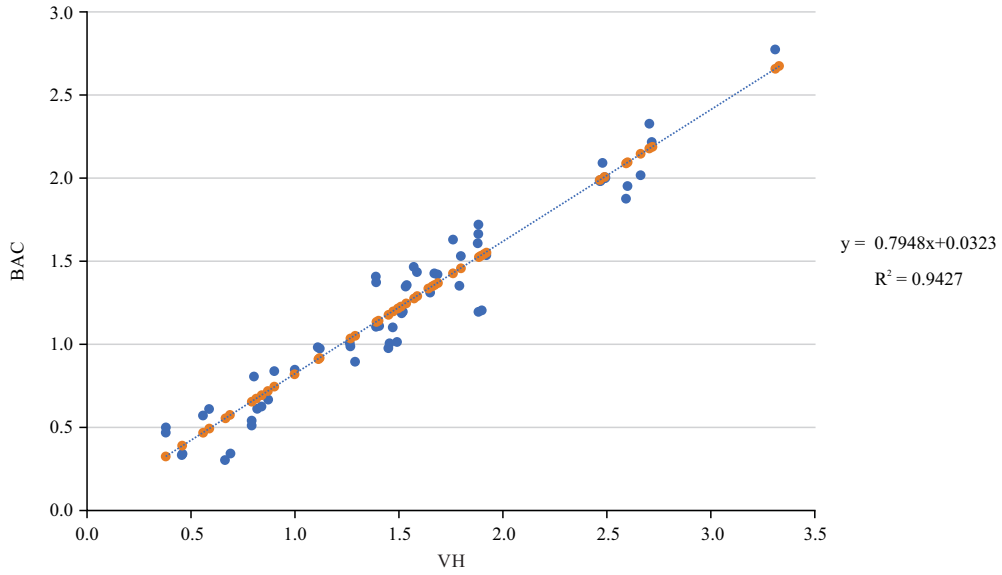

Fig. 4: Linear regression plot between VH (x) and BAC (y) after 180 days

The regression analysis after 180 days yielded the equation:

$$y = 0.7948x+0.0323$$

where, y corresponds to BAC and x to VH ethanol levels. The coefficient of determination (R^2) was calculated at 0.9427 (Fig. 4), $F = 1.051e^{-37}$.

The results indicate the presence of direct proportionality between VH and BAC and high R^2 values show that linear model can be applied when trying to calculate BAC from VH concentrations.

DISCUSSION

Analysing the stability of sampled ethanol during storage in forensic, clinical and toxicology laboratories is of great importance, whether because there is a court request to verify the validity of the analyses, or if the initial results were close to the statutory alcohol limits for driving.

Ethanol levels may decrease in biological samples for several reasons such as leakage in the container or biochemical degradation due to omission of preservative (commonly used one is sodium fluoride)[29]. Specimens can become contaminated either by yeasts or bacteria for various preanalytical reasons which can result in either production or degradation of ethanol. Some studies indicated that there is no difference in whether a preserving agent was used if a specimen was obtained by sterile syringes, with no usage of ethanol as a disinfectant and if samples were kept refrigerated. There is always a question of an analytical method imprecision which was pointed out by multiple authors[23,29,30]. The level of BAC changes during storage time is in relation to initial BAC levels[30]. A group of Italian authors presented a formula which deals with ethanol kinetics as a function of time, CA%, temperature and initial concentration and by their conclusion, it could be used to predict ethanol concentration at any given time during storage[31]. Some authors stated that repeated opening of tubes with blood samples can lead to greater ethanol decrease, suggesting that ventilation between CA% and ambient air can lead to degradation[18], which authors disputed as a plausible explanation[32,33]. Some works implied that CA% is of great importance and tried to determine that CA% is in a direct correlation to degree of ethanol loss[19]. Their explanation was that alcohol can only be lost due to ventilation between liquid and gas phases. Non-enzymatic oxidation reaction by oxyhaemoglobin within erythrocytes can also result in alcohol decay, which is not dependent on CA%[18].

Vitreous is a very useful specimen for post-mortem ethanol analyses. There are several advantages to VH over BAC, the main being its high water percentage and anatomically remote location which hampers contamination of bacteria from the gut and blood. This is especially important when dealing with partially decomposed bodies, or victims of severe trauma[34]. Multiple studies have shown that the ratio between VH and BAC is dependent on water distribution in these specimens and is somewhere in between 1.15 and 1.20:1 with high correlation coefficients. According to the same studies formulas and regression analyses were proposed to calculate BAC when VH is known[35-39]. In a study of 672 forensic autopsies VH ethanol concentration was compared to that of the femoral venous blood. The results show that Pearson's correlation coefficient was r = 0.979 and the mean VH/BAC ratio 1.19:1 standard deviation-SD being 0.285) and this translates into a 95% range ($\bar{\chi} \pm 1.96 \times SD$) of 0.63-1.75[35]. In another large study which included results from 349 autopsies, researchers have also found a high correlation between VH alcohol and BAC. As in the previous study, SD was fairly big, 0.26 mg/mL, which suggests that BAC could be predicted from the results of VH analysis within ±0.51 mg/mL in 95% of cases from the same population[36]. In a publication from 2005, a group of American authors also found a high correlation (r = 0.958) between VH and BAC and the mean VH/blood ratio was 1.24:1[37]. A group of polish authors found no significant differences between the concentration of ethanol in VH and FB, where spearman's correlation was r = 0.96, p<0.01[38]. In one of the most recent publications from the field, a group of Italian researchers found high coefficient of determination between two samples, calculated by linear regression analysis ($R^2 = 0.9981$). The authors confirmed that there was no significant differences between BAC and VH and they concluded that VH could be used as a valid alternative to whole blood samples in those cases where blood is not available[39]. Ethanol in VH samples is fairly stable if kept at low temperatures (4°C) and if fluoride preservative is added[40]. In a different study this was presented as a comparison between ethanol decrease in VH samples with and without preservative and comparing VH ethanol samples with BAC samples stability. In both cases, the changes were highly significant (p<0.001)[41].

The study found that during 180 days of refrigerated storage, there was a significant decrease in BAC and VH ethanol levels. According to previously cited studies, no preservative has added[29], thus mimicking the routine procedure in our institution, which doesn't require preservative adding. This study also found that VH ethanol was much more stable than BAC (p<0.001). Linear regression formulas applied to the results of our study show that a decrease in BAC and VH during storage is dependent on the initial concentrations, but initial levels are not the only factor that influences the degree of changes during time. Because of this, the current study concluded that one is not advised to estimate ethanol loss during storage by the use of the regression equation, nor to calculate initial BAC.

In present study mean VH/BAC was $\bar{\chi} = 1.161$, SD = 0.17 in initial analysis and $\bar{\chi} = 1.244$, SD = 0.223 after 180 days of refrigerated storage. This means that 95% of same population samples fall between VH/BAC ratio of 0.828 and 1.494 for initial analysis and 0.807 to 1.681 after six months ($\bar{\chi} \pm 1.96 \times SD$). These results corresponded to previous, above mentioned studies a linear regression formula (y = mx+b) was calculated as well as coefficient of determination (R^2) between VH and BAC, in an attempt to propose a valid calculation of BAC when VH is known. Statistical analysis shows a strong causative relation between the concentration of ethanol in blood and vitreous humour, practically without any statistically significant difference. This confirms the results of

studies by Szeremeta *et al.*[38] and Savini *et al.*[39], but also confirmed, bearing in mind VH/BAC ratio and SD values, that there are wide individual variations in VH/BAC ratio, so it is not recommended to use regression analysis formula to calculate BAC from VH or vice-versa.

CONCLUSION

There was a significant decrease in BAC and VH ethanol concentrations during six months and significantly more so in blood samples compared to VH, which was to be expected. Although there is a causative relation between the average decrease in ethanol concentration and initial BAC/VH concentrations, it is not the only factor that influences the degree of changes during time, so based on a linear regression it is not possible to estimate the ethanol loss during storage, nor to calculate initial BAC.

SIGNIFICANCE STATEMENT

The purpose of this study was to examine the stability of ethanol in postmortem specimens of blood and vitreous humour in order to determine if there were any significant changes in ethanol concentration and which sample type is more stable in given conditions. A significant decrease was found in BAC and VH levels after 180 days of storage, a positive relation between initial concentrations in blood and vitreous and their changes after 180 days and highly positive relation between VH and BAC levels, both initially and after 6 months. These findings provide knowledge about the stability of drugs and metabolites in biological specimens is of the utmost importance when the results are to be evaluated and/or interpreted.

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