

## STABILITY OF ETHANOL IN BLOOD AND URINE SAMPLES

### STABILNOST ETANOLA U UZORCIMA KRVI I URINA

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**Summary:** The changes of ethanol concentrations in whole blood and urine samples were analyzed depending on temperatures and duration of storage. The aim of the study was to establish standards for the Institute laboratory. Samples of whole blood and urine, taken from drivers with excessive alcohol concentrations (6 groups, 15 samples per each), were analyzed upon delivery and then after storage during different time intervals and at different temperatures. The results showed that alcohol concentrations were significantly reduced with the increase of temperature and prolongation of storage. Only the whole blood samples stored for up to one month at  $-20^{\circ}\text{C}$  did not show significant changes. Room temperature storage of samples is the least suitable way of keeping them, independently of the duration of storage. Urines are not less reliable samples than blood. There are no ethanol differences between blood samples with and without sodium fluoride.

**Keywords:** ethanol stability, sample storage, temperature effect

### Introduction

Based on data from the relevant literature, about 90% of people occasionally consume alcohol (ethanol) in various forms, 40–50% of people have occasional problems as a consequence of alcohol consumption, while 10% of men and 3% of women are addicted to alcohol. The results of such statistics are frequently occurring injuries in traffic accidents, at work, driving under the influence of alcohol, sometimes fatalities (1). Determination of alcohol concentrations is often a compulsory test for those involved in such events and it is important evidence in the court

**Kratak sadržaj:** Ispitivane su promene koncentracije etanola u uzorcima pune krvi i urina u zavisnosti od promene temperature i dužine čuvanja uzoraka, a u cilju postavljanja protokola prijema uzoraka za analizu u laboratoriji Zavoda. Uzorci uzeti od alkoholisanih učesnika saobraćaja analizirani su po dopremanju u laboratoriju, a zatim i posle čuvanja u različitim vremenskim intervalima i na različitim temperaturama (6 grupa po 15 uzoraka). Iz rezultata se uočava da se koncentracije etanola značajno snižavaju sa povećanjem temperature, kao i sa produžavanjem vremena čuvanja. Jedino u uzorcima pune krvi čuvanim do mesec dana na temperaturi od  $-20^{\circ}\text{C}$  nije bilo značajnih promena. Uočava se da je čuvanje uzoraka na sobnoj temperaturi najmanje pouzdano, nezavisno od vremenskog intervala. Urini nisu manje pouzdani uzorci od krvi. Nije uočena razlika u promenama etanola između uzoraka krvi sa i bez natrijum fluorida.

**Ključne reči:** stabilnost etanola, čuvanje uzoraka, uticaj temperature

procedure. Results of the determination are often the deciding factor between innocence and guilt, so these values must be accurate.

The most frequent samples used by biochemical laboratories to determine ethanol concentrations are the whole blood, serum and urine. Standardization of sample-taking requirements has been achieved to a great extent by training the staff and by the use of commercial vacuum tubes with or without anticoagulants and preservatives. No matter which body fluid is concerned, there are still big practical problems referring to the transport of sensitive samples coming from outside, and storage of samples before and after the determination of ethanol. The preanalytic phase has been recognized to have a substantial role in the quality and reliability of ethanol analytical results, which very much depend on the type and quality of specimens provided. Proper collection, handling and storage of the blood ethanol specimens are essential in medicolegal cases involving the question of sobriety.

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Also, a standard operating procedure is necessary to ensure the maximum reliability.

Prescribed standards and laws do not provide clear instructions for the samples storage. Laboratories have to define them according to their experience and needs.

The aim of this study was to analyze the changes of ethanol concentrations in whole blood and urine depending on temperatures and duration of sample storage and, based on the results obtained, to establish the standards for the laboratory of the Institute.

## Materials and Methods

Whole blood and urine samples taken from the drinking drivers were tested for the presence of ethanol. Blood and urine were delivered to the laboratory of the Health Care Institute for the Employees of the Ministry of Internal Affairs by a courier post. Materials were sampled in different health institutes in several regions throughout Serbia applying the recommended procedure. Conditions of sample taking, storage and transport could not be fully controlled. The blood was taken with anticoagulants (KEDTA and Fluoride/Oxalate), and urine samples were taken without any additions. Initial ethanol values in all samples were determined within 24 hours from the receipt of materials. The selected samples were those with ethanol concentrations higher than 0.5 g/L (the allowed limit value of ethanol presence in motorists in our country).

The samples (120 in total) were stored at different temperatures and for different periods, and then the ethanol concentrations were again determined according to the following schedule (15 samples each): Group 1, 7 and 14 days at room temperature (G1); Group 2, 1–3 months at +4 °C (G2); Group 3, more than 3 months at +4 °C (G3); Group 4, 1–3 months at –20 °C (G4); Group 5, more than 6 months at –20 °C (G5); Group 6 (urine samples), more than one month at +4 °C (G6). Two groups of samples taken with KEDTA and Fluoride/Oxalate and stored for up to 3 months at +4 °C were specifically compared. Thus formation of groups was conditioned by the Institute laboratory needs and the issues which had to be solved.

Ethanol concentrations were determined by using a Roche commercial test with the alcohol dehy-

drogenase (EC 1.1.1.1) (2), on the IL600 System (Instrumentation Laboratory). Tests were performed in duplicate and expressed in g/L (‰), in the way as they are used in practice.

The results were presented as mean ± standard deviation (SD). Group differences were tested by the analysis of variance and Student-t test. A statistical significance was accepted if  $p < 0.05$ .

## Results

The *Table 1* shows mean values of ethanol per group and their relative decreases.

Test results show that a decline in ethanol concentrations occurred in all groups, at different levels.

The most significant changes were found in G1 ( $p < 0.001$ ), where the value decline for 14 days was 22.4%. G2 showed a significant decline, 95.8%, of the initial value, ( $p < 0.01$ ), as well as G3, 92.1% ( $p < 0.001$ ). However, G4 did not show any statistically relevant changes, having a 4% decrease in value. G5 maintained 85% of the initial ethanol values, with a significant decline ( $p < 0.001$ ). Changes in the urine were statistically relevant ( $p < 0.05$ ), 90% of initial concentrations. When comparing the changes in samples taken with Fluoride/Oxalate and without preservatives, with KEDTA, no significant differences were obtained, and the decline in ethanol concentrations in the tested groups was almost identical (2.3 and 2.4%, respectively).

## Discussion

Preanalytical preparation and problems associated with the long-term storage of samples taken from living people in order to determine ethanol concentrations have rarely been discussed in literature and therefore date back in the past (3–5). More commonly, such researches refer to forensic samples. It is thought that the quality of post-mortem samples deteriorates faster than the quality of samples from living people, primarily due to the presence of bacteria, yeast and fungus (6). Changes of ethanol concentrations in post-mortem samples were not analyzed in this study.

The temperature of storage, duration of storage, selection of preservatives and air quantity above the sample are said to be the most common causes of

**Table 1** The values of ethanol (‰) and relative decreases in the studied groups.

	G 1		G 2	G 3	G 4	G 5	G 6
	7 days	14 days					
Ethanol, before	1.52 ± 0.29		1.68 ± 0.74	1.53 ± 0.36	1.74 ± 0.98	2.60 ± 1.16	2.11 ± 1.60
Ethanol, after	1.39 ± 0.31	1.18 ± 0.35	1.61 ± 0.65	1.41 ± 0.34	1.67 ± 0.95	2.21 ± 0.91	1.90 ± 1.38
Decline, %	7.9	22.4	4.2	7.9	4.0	15.0	10.0
Probability, p	<0.001	<0.001	<0.01	<0.001	NS	<0.001	<0.05

changes in the value of ethanol in whole blood samples. There is the synergism of these influences and it is hard to discuss the conditions separately.

Due to rather specific and not enough controlled conditions of storing the samples before they are received for analysis, the main aim of our study was to analyze the influences of temperature and duration of storage. The results of this research clearly show that the most significant changes in the values of ethanol were caused by the surrounding temperature. Storage of samples for up to 7 days resulted in a significant decline of value, by 7.9%, and for up to 14 days even by 22.4%, what none of other conditions had caused. By lowering the temperature of storage, the level of ethanol loss decreases, and if the samples are frozen at  $-20^{\circ}\text{C}$ , this decline is insignificant for the period of at least one month. A long-term storage at the same temperature is not safe. The urine is a more sensitive biological material and changes in ethanol concentrations are rapid (7–9). Storage of whole blood and urine samples at room temperature is surely the least reliable way. The results indicate that it is necessary to freeze the samples as soon as possible after assays and possible re-assays if they are to be stored for a longer period.

The results show that the duration of sample storage cannot be considered independently from the temperature influence and that it has a dominating effect on the outcomes.

The results similar to ours were obtained in testing the storage of whole blood and serum at temperatures ranging from  $26.7$  to  $37.8^{\circ}\text{C}$ , when Winek et al. concluded that the loss of ethanol is much higher in the whole blood than in serum, amounting to 10–19% already in the period of 35 days (10). It is thought that the loss occurs mostly because of the oxidation of ethanol and less because of evaporation.

Tests of temperature influences differ in the selection of criteria (3, 4, 10), and therefore the use of results is of limited value. They are usually accompanied with the suggestion that own criteria should be established.

Together with the temperature influence, the influence of preservatives is often considered (by general consensus it is sodium fluoride). Its presence inhibits glucose, growth of microbes and subsequent formation of ethanol as a consequence of the activities of microbes, most often *Candida albicans* (8, 11, 12). Lewis (13) suggests that with the addition of 1% sodium fluoride, there was no significant increase in ethanol concentration at either temperature. At the same time, there are, however, at least two often quoted reports concluding that sodium fluoride may be ineffective for the prevention of ethanol formation in blood samples containing sufficiently high concentrations of *Candida albicans* (14, 15).

The results obtained in our research, which was

conducted on a small number of samples and in which only the storage for up to 3 months at  $+4^{\circ}\text{C}$  was considered, did not give preference to blood sampling in tubes containing sodium fluoride. A decline in ethanol concentrations was almost the same as in tubes without a preservative. It might be necessary to investigate the differences between the effect of sodium fluoride on the preservation of post-mortem samples and those taken from living persons.

This research did not evaluate the influence of decreased concentrations of ethanol caused by oxidation due to presence of large amounts of air above the sample, but this particular cause of changes can primarily relate to the urine samples because of the way of sampling. Ferrari et al. (16) found that the ethanol value decreased by 33% in samples with 35% air, stored for over 15 days at  $25^{\circ}\text{C}$ . The recommended air content is 0%, whenever it is possible.

In view of the results obtained in this research and recommendations available in literature, we can say that each laboratory should establish its own limits of reliable storage given the actual conditions in that laboratory.

The conditions of collecting whole blood and urine samples for the laboratory of the Institute brought about the need for writing a recommendation for their proper sampling and transport. The recommendation has been based on the relevant legislation and experience so far, and it is intended for the field health-care facilities and for the officials who give orders to check intoxication of certain persons. Moreover, the results of this research helped to establish the rules of storing and handling biological materials required for the determination of ethanol concentrations:

1. Either unpreserved urine samples or whole blood samples taken with an anticoagulant can be equally used, as well as those with or without a preservative (sodium fluoride).
2. Determination of ethanol concentrations should be done within 24 hours, in duplicate.
3. Analyzed samples should be divided into two portions, transferred to the appropriate sterile, well sealed dishes, with as small as possible amount of air above the sample.
4. It is desirable to store samples at the temperature of  $-20^{\circ}\text{C}$ , for up to one year.

A long-term storage of samples is rarely needed for possible expertise, or due to the Court's order, but more commonly to check the identity of a person whose blood has been tested.

Although it is impossible to eliminate all interfering factors or influences occurring during the preanalytic phase, their consideration should facilitate the assessment of sample quality and the analytical result obtained from the sample.

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