Stability of Postmortem Blood Ethanol under Experimental Conditions

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ABSTRACT

Objective: To do analysis of ethanol in postmortem blood samples stored at different temperatures and time intervals and to compare ethanol concentration in blood samples with high and normal glucose amounts when stored at room temperature.
Methods: Blood samples collected from the fifty autopsy cases with 1% sodium fluoride as preservative were analyzed immediately by headspace gas chromatography. Each blood sample was divided into three sets. Each set of blood samples were stored at room temperature, 4°C and -20°C, respectively. The storage samples were analyzed for ethanol concentration at different time interval at: 2, 4, 7, 14, 28, 60 and 90 days after collection. Each of ten blood samples of normal (70-110 mg/dl) and high (greater than 200 mg/dl) glucose level were tested for ethanol concentration immediately and then transferred to twelve polypropylene tubes with a tightened cap. The blood sample was stored at room temperature and analyzed for various time intervals including 2, 4, 7, 14 and 28 days.
Results: Ethanol concentrations in blood stored at 4°C and -20°C were stable up to 14 and 7 days before slowly declining until 90 days. Whereas blood samples stored at room temperature the ethanol concentrations continuously decreased throughout storage time and was less stable at any storage time than those stored at 4°C and -20°C. Ethanol concentrations in the high glucose blood group were significantly increased after 7 days while those in the normal glucose blood group were mostly unchanged.
Conclusion: The stability of ethanol concentration in preserved blood stored at 4°C was longer than those stored at -20°C and room temperature. A high glucose level may lead to increased ethanol concentration when stored at room temperature.

Keywords: Ethanol; headspace gas chromatography; postmortem blood

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Ethanol analysis is the most common and routine test performed on forensic specimens.[1] The determination of ethanol in blood samples and its interpretation are essential in the medico-legal aspect. This is important in court proceedings relative to the interpretation of blood ethanol concentration. Interpretations of blood ethanol concentration are often confronted by postmortem production or loss of ethanol.[2] The important factors were found to be temperature, sodium fluorideconcentration and time of storage.[3] Studies have shown that ethanol can be generated by bacterial synthesis in vitro. Endogenous ethanol is a result of microbial activity, primarily on glucose.[4,5] Glycolysis is considered the principle process for ethanol production but some other metabolic processes are also possible using glucose as a substrate. The probability of postmortem ethanol synthesis increases with the storage temperature and the time interval before analysis was done.[6] However; Winek et al found ethanol loss from the whole blood sample in bacterial contaminated blood specimens.[7] They also studied the effect of storage on blood alcohol concentration at elevated temperatures and found that the alcohol loss from whole blood samples may be attributed to chemical oxidation rather than to elevated temperatures.

Data on the preservative of ethanol in blood specimen is fragmented in numerous reports and different study designs have been applied.[8] Sodium fluoride can inhibit the activity of phosphoglucomutase and the metabolic process of microbes except in cases of high glucose and *Candida spp.*[9] 1% sodium fluoride is the most appropriate amount of preservative for ethanol blood analysis.[10] Some others tried to add another preservative but these were still less useful for this analyte.[6,11]

The first purpose of this study is to determine the stability of ethanol in postmortem blood stored under various conditions by using 1% sodium fluoride as a preservative and examine the preservation of ethanol over

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a period of 3 months to determine whether the stability increases or decreases at a greater rate after storage. The second study is set for observing the effect of glucose on blood ethanol concentration, especially in cases of diabetic diseases.

MATERIALS AND METHODS

Reagent

The stock ethanol solution was 1,580 mg/dl ethanol in water. The working control standard was prepared to 50 mg/dl with double distillation deionized water (DDW). The stock internal standard (I.S.) was 10% v/v in DDW, and was prepared from AR grade 2-propanol. Working I.S. prepared to 1 %v/v with DDW. All aqueous solution was prepared using double DDW, which was obtained by using a water purification system (Human Power III plus, Human Corporation, Jamsil Bon-Dong, Songpa-Ku, Seoul, Korea).

Head Space Method

Head space chromatographic (Hs.) determination for ethanol and other volatile substances were analyzed by a Headspace Sampler (TurboMatrix40, Perkin-Elmer, Norwalk, CT, USA) equipped with gas chromatography.The headspace parameter settings: the vial heater, needle and transfer line was held at 50°C, 60°C and 70°C, respectively. Carrier flow was 18.1 psi, 5 minute cycle time and thermostat time was set at 15 minutes.

GC method

A Perkin-Elmer Gas chromatograph (GC) equipped with a flame ionization detector (FID) was used for ethanol determination. Column was RTX®-BAC 1 (Restek Corporation, Bellafonte, USA) 30 meters long 0.32 mmID, 1.8 umdf and maximum temperature was 240°C. Both GC injector and detector were set at 200°C. The GC oven was isothermally held at 40°C for the duration of 5 minutes run. The carrier gas was Nitrogen and held at 11.0 psi. Hydrogen and Air Zero were set at 45.0 ml/min and 450 ml/min, respectively.

Quantitation

The area ratio of ethanol and other volatile substances to the internal standard of the specimen (blood) were compared by a calibrator and verified with commercial controls. The limit of detection, limit of quantifation and linearity range were evaluated. Results greater than the linearity range were reported for greater and lower linearity range. Ethanol concentration was operated by a Turbochrom Workstation Version 6.1.2.

Procedure

Part I

Thirty milliliters (ml) of heart blood sample were obtained by sterile syringe at autopsy and kept in a 1.0% sodium fluoride polypropylene tube. All fifty cases of blood were tested by Hs-GC for ethanol concentration immediately (T_0) then transferred to 21 polypropylene tubes with a tightened cap. The blood sample was stored at three different temperatures (room temperature, 4°C and -20°C) and analyzed for various time intervals including 2, 4, 7, 14, 28, 60 and 90 days.

Part II

Blood glucose was determined by the One Touch Ultra: Blood Glucose Monitoring System for glucose determination (LIFESCAN, Milpitas, CA, USA), according to the package insert instructions.[12] All 10 cases of blood in each group of normal (70-110 mg/dl) and high (greater than 200 mg/dl) glucose levels were tested by Hs-GC for ethanol concentration immediately (T_0) and then transferred to twelve polypropylene tubes with tightened cap. The blood sample was stored at room temperature and analyzed for various time intervals including 2, 4, 7, 14, 28, 60 and 90 days.

Statistic analysis

All data were compared to the ethanol concentration at T_0 (100%) as percentage and were presented as mean and standard deviation (mean ± SD). A general linear model with repeated measures was used to determine the difference among the timings. Post hoc comparisons were made using the LSD test. One way analysis of variance (ANOVA) was used for comparison among the temperature groups and the significant difference between the two mean values was evaluated by the LSD test. A difference was considered significant at $p<0.05$.

RESULTS

Method validations were performed before ethanol analysis. The limit of detection and limit of quantization were 1.0 mg/dl and 3.0 mg/dl, respectively. Precision and Accuracy were also done. These values were 5% RSD (Relative Standard Deviation) and 49.3-50.6 mg/dl for 50 mg/dl of ethanol standard. The linearity range was 5-400 mg/dl which covered the analysis of ethanol.

Part I. To determine the stability of ethanol in blood stored under various conditions

The ethanol concentration in blood samples of all fifty cases were detected at once. Because the results were wildly different in the range from 42 to 285 mg%, the data were transformed to the percentage of ethanol recovery. The result of the stability of ethanol level in stored blood at different temperatures and time intervals is shown in Fig 1.

For blood stored at 4°C, the percentage recovered of ethanol concentration were found to be stable until 14 days when compared to T_0 ($F_{7,416}=18.68$, $p<0.05$) and further decreased slowly until 90 days. For blood stored at -20°C, the percentages of ethanol concentration were presented in the same manner with an early significant decrease of ethanol level at 7 days when compared to T_0, $p<0.05$.

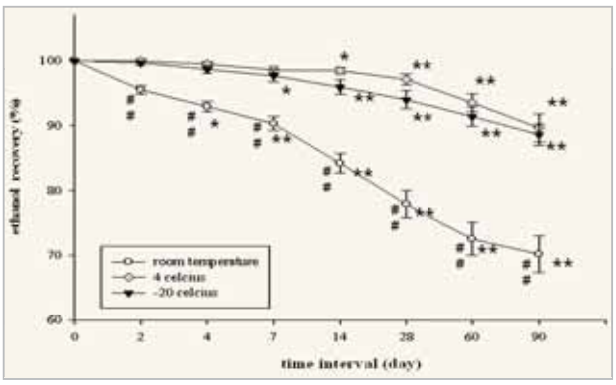

Fig 1. Percentage recovery of ethanol concentrations in preservative bloods held at room temperature, 4°C, and -20°C for different time. Data were mean ± SD of fifty samples in each group.* $p<0.05$, ** $p<0.01$ vs T_0; ## $p<0.01$ vs 4°C.

For blood stored at room temperature, the percentages of ethanol concentration were slightly declined through 7 days and demonstrated a significantly decreased difference from T_0 ($p<0.05$) at 4 days. After 1 week, the results continuously decreased until they reached the lowest values at 90 days (66.74 ± 18.34%) and the values were very low as compared to T_0, $p<0.01$. Therefore, the duration of storage time may have affected the change of stability of ethanol concentration in blood stored at: -20°C, 4°C and room temperature.

In each storage time, the blood ethanol concentration at different temperatures were compared. There were not significantly different between the ethanol concentrations in blood stored at -20°C and 4°C at any storage time. At day 2 through day 90, the percentage of ethanol concentrations in blood stored at room temperature clearly demonstrated a lower result than at 4°C ($p<0.01$) and extremely significant following the passage of time. Therefore, these results indicate that temperature could affect the stability of ethanol concentration in blood at any storage time.

Part II. To study the effect of glucose on blood ethanol concentrations

The study in this part is to compare the stability of ethanol in unpreserved blood with high (greater than 200 mg/dl) and normal (70-110 mg/dl) glucose concentration stored at room temperature at different durations: at once, 2, 4, 7, 14, 21 and 28 days. The results are shown in Fig 2.

The percentages of ethanol concentration in blood with a high glucose level were gradually increased until they showed significant elevation at 7 days when compared to T_0 ($F_{4,45}=5.68$, $p<0.01$) and reached the maximum-increased value at 14 days and remained at the highest value until 28 days. While the percentages of ethanol level in blood with normal glucose concentration were not significantly changed from T_0, during the storage time for up to 28 days except day 7 which was significantly increased ($F_{4,45}=2.99$, $p<0.05$) when compared to T_0.

For comparison between groups of glucose levels it demonstrated that the percentage elevation of ethanol level from the high glucose concentration showed a significant difference from the group of normal glucose concentration ($p<0.05$) when stored up to 7 days and a more significant difference ($p<0.01$) in all storage times after 7 days. These results thus indicate that the increased value of ethanol stored up to 7 days time interval revealed the most significant difference for both groups. The blood samples with high glucose content revealed increased ethanol level compared to those with normal glucose content when stored after 7 days.

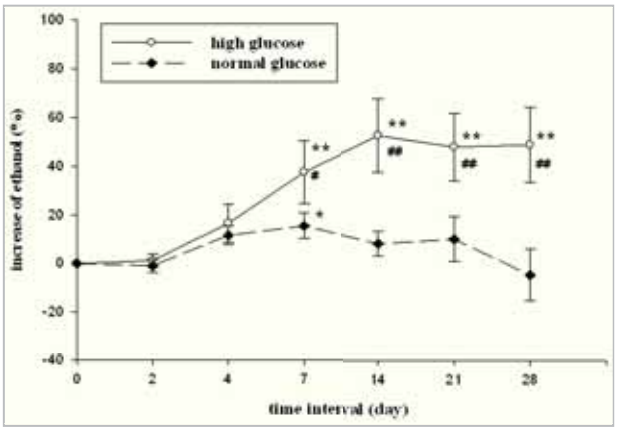

Fig 2. Stability of ethanol concentrations in no preservative bloods with high and normal glucose levels at room temperature. Data were mean ± SD of ten samples in each group. * $p<0.05$, ** $p<0.01$ vs T_0; # $p<0.05$, ## $p<0.01$ vs normal glucose level.

DISCUSSION

The determination of alcohol in biological specimens is the most common analytical procedure for legal purposes. The biological specimens submitted for routine alcohol analysis are usually blood and/or urine. Ethanol can be either increased or decreased when stored in different conditions. Fermentation seems to be the most responsible for an elevated ethanol level in blood and tissues.[4,5,13] This effect can be diminished by adding 1% sodium fluoride as a preservative. Amick et al showed that when samples collected in a tube containing sodium fluoride and stored at 4°C or 25°C ethanol production by *Saccharomyces cerevisiae*, they are inhibited.[14] Many authors proposed that ethanol was decreased rather than elevated.[7,15,16] A decrease in ethanol concentration was dependent on several factors including storage time, storage temperature, concentration of preservative, ethanol concentration and types of container.[17] However, the important variables were only storage time, storage temperature and concentration of sodium fluoride.

This study was emphasized to analyze ethanol for stability in laboratory work. The result showed that ethanol in blood with 1% sodium fluoride as preservative stored at 4°C was most stable when compared to those stored at -20°C and room temperature. This study supported the results of a previous study that alcohol in refrigerated blood revealed better stability than in the deep freeze and at room temperature.[18]

It is known that ethanol can be degraded when storage for analysis, mostly due to the oxidation of ethanol to acetaldehyde.[19] The important mechanism of stability of alcohol in stored blood was strongly temperature-dependent and the alcohol oxidation reaction was not inhibited by sodium fluoride. This alcohol oxidizing activity was found to reside in the red blood cells.[16] It, therefore, appears that the loss of ethanol in stored whole blood sample was due to the chemical oxidation rather than the physical loss. The blood containers have to be effectively sealed, a preservative such as 1% sodium fluoride added to prevent microbial growth, and the ethanol oxidation mechanism has to be blocked with an inhibitor, sodium fluoride being ineffective in this respect.

It was found that storage of blood samples at room temperature was mostly decreased when compared to other temperatures. Previous study has reported that ethanol can be lost up to 19 % in 35 days time interval when stored at room temperature (26.7°C).[7] Because alcohol oxidization is temperature-dependent, ethanol levels at 4°C and -20°C were slightly decreased when compared to storage at room temperature. For all of the time intervals, the ethanol level continuously decreased and this effect is believed to diminish false positives for ethanol analysis and the ethanol level below the cut-off value would not be able to be above this value when stored under these conditions. In this study we found the pattern for the decreasing of ethanol at various time intervals and temperatures, but the number of cases was not enough to get the arithmetic relationship for the estimation of ethanol decrease when time interval and temperature were known. However, this appearance

may also be applied to other samples for stability of ethanol and other volatile substances.

The effect of glucose on ethanol in stored-blood samples, tissues and other body fluids was similar to previously studies.[9,14,20] The results of the studies were adopted for blood alcohol preservation in forensic laboratories. They found that unpreserved samples which were supplemented with both yeast and glucose attained ethanol, but ethanol could not be detected in any corresponding duplicating samples, which were preserved with 1% sodium fluoride.

In forensic cases we found that ethanol analysis was problematic in DM and occasionally putrefied cases. This study was set up for preliminary observation of the effect of glucose on the ethanol analysis without any preservative. Glucose determination was done after ethanol determination every 2, 4, 7, 14, 21 and 28 days of storage time (data not shown). We found that 7 days time interval revealed elevated ethanol level for 2 times of the original level. The mechanism of the elevation of ethanol is believed to be the fermentation of blood samples in this condition. The decreases of glucose amounts were related to the increase of ethanol levels. The high glucose level affected the amount of ethanol more than the normal glucose level. This finding supported the results of previous studies for the effect of glucose on increasing ethanol concentration.

Without any preservative, ethanol concentration could be elevated in stored blood at room temperature. We did not mention in stored blood at cool place such as 0°C, 4°C and -20°C because many literatures confirmed that ethanol production is temperature dependent.[20] The glucose amount in blood stored at room temperature was significantly decreased at 7 days of storage time interval, while ethanol showed significant increase at 7 days. This observation supports the previous study with a good correlation. Bogusz et al reported that the maximum increase of ethanol in blood stored at 20 ± 2°C occurred at 5 days and the maximum decrease of glucose also presented at 5 days time interval.[5] The different results of time interval may be due to the difference in room temperature. These findings seem to be reasonable for the effect of glucose on ethanol stability.

In this study, we found that the ethanol level in the cases with high glucose content could be increased more than those with normal glucose content. Therefore, the analysis of ethanol in blood with a high glucose level should be considered when samples were stored for several days or in those of putrefied bodies.

CONCLUSION

The concentrations of ethanol in bloods with 1% sodium fluoride as preservative stored at 4°C were more stable than at -20°C and room temperature. In addition, the ethanol concentrations in blood samples stored at room temperature were continuously decreased throughout storage time and were less stable at any storage time than those stored at 4°C and -20°C. The stability of ethanol concentrations in blood stored at 4°C and -20°C was up to 7 and 14 days. In comparison to normal glucose blood, a high glucose level may induce the elevation of blood ethanol concentration when stored at room temperature.

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