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Decreases in blood ethanol concentrations during storage at 4 °C for 12 months were the same for specimens kept in glass or plastic tubes

A.W. Jones [a,b,*], E. Ericsson [a]

[a] Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine, Linköping, Sweden
[b] Department of Clinical Pharmacology, Medical Faculty, University of Linköping, Sweden

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ABSTRACT

Background: The stability of ethanol was investigated in blood specimens in glass or plastic evacuated tubes after storage in a refrigerator at 4 °C for up to 12 months.
Methods: Sterile blood, from a local hospital, was divided into 50 mL portions and spiked with aqueous ethanol (10% w/v) to give target concentrations of 0.20, 1.00, 2.00 and 3.00 g/L. Ethanol was determined in blood by headspace gas chromatography (HS-GC) with an analytical imprecision of < 3% (coefficient of variation, CV%). Aliquots of blood were re-analysed after 2, 7, 14, 28, 91, 182 and 364 days of storage at 4 °C.
Results: The standard deviation (SD) of analysis by HS-GC was 0.0059 g/L at 0.20 g/L and 0.0342 g/L at 3.00 g/L, corresponding to CVs of 2.9% and 1.1%, respectively. The decreases in blood ethanol content were analytically significant after 14–28 days of storage for both glass and plastic tubes The mean (lowest and highest) loss of ethanol after 12 months storage was 0.111 g/L (0.084–0.129 g/L) for glass tubes and 0.112 g/L (0.088–0.140 g/L) for plastic tubes. The corresponding percentage losses of ethanol were 43–45% at a starting concentration of 0.20 g/L and 3.9–4.1% at 3.00 g/L.
Conclusion: The concentration of ethanol in blood gradually decreases during storage at 4 °C. After 12 months storage the absolute decrease in concentration was ~0.11 g/L when the starting concentration ranged from 0.20 to 3.0 g/L. Decreases in ethanol content were the same for specimens kept in glass or plastic evacuated tubes.

1. Introduction

The first evacuated tubes used to sample venous blood for clinical laboratory analysis appeared in the early 1950s [1]. These tubes were originally made of soda glass, although more recently plastic tubes have become available [2]. Tubes made of plastic are more robust and are less likely to break during transport, handling and storage of specimens or after cycles of freezing and thawing [3]. The switch from glass to plastic tubes has also occurred in connection with forensic analysis of ethanol and other drugs in blood, such as in traffic-law when impaired drivers are arrested [4]. This raises the question of the stability of blood-ethanol concentrations after various periods of storage in glass and plastic evacuated tubes.

For several decades the Swedish National Laboratory of Forensic Toxicology purchased fluoride-oxalate (grey stoppered)

* Corresponding author at: Department of Clinical Pharmacology, Faculty of Health Sciences, Linköping University, Sweden.
E-mail address: Wayne.jones@liu.se (A.W. Jones).

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evacuated tubes from a Belgian company (Terumo Europe N.V.). These tubes were made of glass with a nominal volume of 10 mL and contained sodium fluoride (100 mg) and potassium oxalate (22.5 mg) as preservative and anticoagulant, respectively. Several years ago the manufacturer informed us that they intended to stop selling glass evacuated tubes, but were able to supply plastic tubes containing exactly the same chemical additives.

Venous blood samples from impaired drivers arrested throughout Sweden are sent for analysis of ethanol and other drugs to a central laboratory. The specimens are shipped by express mail and usually arrive within 1–2 days. After registration the blood samples are placed in racks and kept in a refrigerator at 4 °C pending analysis a few days later. In forensic casework, a request to re-analyse the blood specimen is not uncommon, such as if the accuracy of the result is challenged or if there is some doubt about integrity of the sample. This makes it important to know whether the ethanol concentrations in blood are stable during short- and long-term storage of specimens in a refrigerator [5].

The aim of this study was to compare and contrast the stability of ethanol in stored blood samples after storage in sealed evacuated tubes made of soda glass or plastic for up to 12 months.

2. Methods

2.1. Evacuated tubes

For purposes of the present study Terumo Europe N.V. (Leuven, Belgium) supplied us with VENOJECT glass evacuated tubes (product number VT–100SFX07) and VENOSAFE plastic tubes (product number VF–109SFX07). Both the glass and plastic tubes were fitted with grey stoppers and contained a mixture of sodium fluoride (100 mg) and potassium oxalate (12.5 mg) as preservative and anticoagulant, respectively [6].

2.2. Spiking of blood with ethanol

Blood (~500 mL) was purchased from the blood transfusion unit at the University Hospital in Linköping and was used to prepare a range of samples with known target concentrations of ethanol. The bag of blood was rotated several times to ensure mixing of plasma and erythrocytes before aliquots were removed for mixing with ethanol.

Spectroscopic grade ethanol (99.9% purity) was diluted with distilled water to make a 10% w/v solution by weighing on a sensitive balance. Known amounts of the 10% ethanol stock solution were weighed directly into 50 mL graduated flasks and immediately made up to the mark by carefully adding well-mixed blood. In this way target blood ethanol concentrations of 0.20 g/L, 1.00 g/L, 2.00 g/L and 3.00 g/L were prepared.

After spiking the blood with ethanol the target concentrations were verified by triplicate analysis by headspace gas chromatography (HS-GC), a method that was described in more detail elsewhere [7]. The mean concentration of ethanol from analysis of three glass and three plastic tubes of blood was taken as the initial (starting) concentration. Evacuated tubes (10 mL) made of glass or plastic were filled with ~9 mL of spiked blood and immediately made air-tight with the rubber stoppers supplied.

2.3. Gas chromatographic analysis

For routine forensic blood-alcohol analysis two HS-GC auto-sampler instruments (HS–101) were supplied by PerkinElmer Inc., (Waltham, MA, USA). The two instruments were fitted with capillary columns (BAC–1 and BAC–2) purchased from Restek Corporation (Bellefonte, PA, USA). Two laboratory technicians performed all blood–ethanol determinations and they worked independently with different GC instruments and diluters.

The glass and plastic tubes containing blood were placed in a rotamix device for a few minutes before the stoppers were removed and aliquots withdrawn for GC analysis. Dilution of the blood with internal standard was done with equipment purchased from Hamilton Inc., (Reno, NV, USA). The Microlab 503A diluter–dispenser was programmed so that 100 μL of blood was diluted 11 times (1+10) with aqueous n-propanol as the internal standard. The diluted blood samples were ejected into 22 mL glass vials, which were immediately made airtight with a butyl rubber stopper and crimped on aluminium cap. All tubes of blood were analysed in duplicate and the mean result of six determinations (three glass and three plastic tubes) was taken as the best estimate of blood–ethanol concentration after various periods of storage.

2.4. Experimental design

To simulate conditions that might operate during transport of specimens to the laboratory, for the first two days of storage one plastic and one glass tubes was in a horizontal position and two other tubes stood upright (vertically) in a rack. After the first 2 days of storage all tubes were positioned upright (vertically) in racks and kept in a refrigerator at 4 °C pending re-analysis. After designated storage times of 7, 14, 28, 91, 182 and 364 days the racks of blood were removed from the refrigerator and allowed to attain room temperature before aliquots were removed for GC analysis as described above.

Table 1
Analytical imprecision profile, standard deviation (SD) and coefficient of variation (CV) for blood ethanol determination by headspace gas chromatography. Also shown are critical differences that need to be exceeded to give an analytically significant change for means of 2 or 6 determinations.

Blood ethanol concentration (g/L)	SD (g/L) (CV, %)[a]	Critical difference (g/L) Mean of 2 determinations	Critical difference (g/L) Mean of 6 determinations
0.20	0.0059 (2.9)	0.0116	0.0067
1.00	0.0137 (1.4)	0.0268	0.0155
2.00	0.0241 (1.2)	0.0472	0.0273
3.00	0.0342 (1.1)	0.0679	0.0387

[a] SD and CV refer to analytical imprecision of single determinations based on several thousand duplicate analyses.

3. Results

3.1. Analytical precision

Table 1 shows the precision profile of blood–ethanol analysis by HS-GC derived from making thousands of duplicate determinations. The standard deviation (SD) of a single determination increased with the concentration of ethanol in blood, whereas the coefficient of variation [(SD/mean) × 100] was more constant over the range 0.20–3.00 g/L. Also shown in Table 1 are critical differences in ethanol concentration that must be exceeded before a change (increase or decrease) is considered analytically significant [5]. For this particular stability study, the mean of six determinations was used because blood in three glass and three plastic tubes was analysed.

If the change in concentration of ethanol in blood from the glass and plastic tubes exceeds the values given in Table 1, then this indicates analytically significant changes in concentration have occurred. This statistical procedure of evaluating critical differences to assess change from initial value has been described elsewhere [8,9]. The change in mean ethanol concentration needed to reach analytical significance depends on the number of replicate determinations and is calculated as follows:

$$\text{Critical difference (mean of 2 determinations)} = 1.96 \times \sqrt{2} \times [SD/\sqrt{2}]$$

$$\text{Critical difference (mean of 6 determinations)} = 1.96 \times \sqrt{2} \times [SD/\sqrt{6}]$$

where SD is the standard deviation of a single determination and 1.96 corresponds to the 95% percentile point for the area under a normal error distribution.

3.2. Changes in ethanol content during storage

The results in Table 2 show no influence of keeping tubes vertically or horizontally for two days at room temperature before analysis. This was confirmed whether blood samples were kept in glass or plastic tubes.

Table 3 shows the rate of decrease in blood ethanol concentrations for blood kept in glass or plastic tubes and stored in a refrigerator at 4 °C for up to 12 months. The decreases in ethanol concentration during storage did not depend on whether blood was kept in glass or plastic tubes. The decreases in ethanol content were not analytically significant until after 14 days of storage (samples with concentration 0.20 g/L and 1.0 g/L) and 28 days (samples with concentration 2.0 g/L and 3.0 g/L) for both glass and plastic tubes.

The mean (lowest and highest) losses of ethanol after 12 months storage were 0.111 g/L (0.084–0.129 g/L) for glass tubes

Table 2
Comparison of blood ethanol concentrations (g/L) in glass or plastic tubes with fluoride-oxalate preservatives when the tubes were positioned vertically or horizontally at room temperature for two days.

Tube position	Tube material glass (G) or plastic (P)	Ethanol conc. 0.195 g/L	Ethanol conc. 1.016 g/L	Ethanol conc. 2.020 g/L	Ethanol conc. 3.019 g/L
Horizontal (one tube)[1]	Glass tube	0.191	1.010	2.026	3.029
	Plastic tube	0.190	1.013	2.013	3.017
	G-P Diff	0.001[2]	−0.003[2]	0.013[2]	0.012[2]
Vertical (two tubes)[3]	Glass tube	0.191	1.014	2.023	3.027
	Plastic tube	0.192	1.014	2.012	3.029
	G-P Diff	−0.001[2]	0.000[2]	0.0011[2]	−0.002[2]

[a] Duplicate determinations on a single tube.
[b] No analytically significant difference.
[c] Duplicate determinations on two tubes.

Table 3
Changes in blood–ethanol concentrations (g/L) for specimens kept in glass (G) or plastic (P) tubes containing fluoride-oxalate as preservatives when these were re-analysed after storage at 4 °C for up to 12 months.

Days of storage	Tube material	Ethanol conc. 0.195 g/L	Ethanol conc. 1.016 g/L	Ethanol conc. 2.020 g/L	Ethanol conc. 3.019 g/L
2	Glass tube	0.191	1.013	2.025	3.028
	Plastic tube	0.192	1.014	2.012	3.025
	G-P Diff.[a]	−0.001[b]	−0.001[b]	0.013[b]	0.003[b]
7	Glass tube	0.188	1.004	1.995	2.997
	Plastic tube	0.188	1.005	1.999	2.989
	G-P Diff.[a]	−0.000[b]	−0.001[b]	−0.004[b]	0.008[b]
14	Glass tube	0.179	0.994	1.999	3.004
	Plastic tube	0.181	0.995	1.988	2.993
	G-P Diff.[a]	−0.002[b]	−0.001[b]	0.011[b]	0.011[b]
28	Glass tube	0.175	0.985	1.971	2.975
	Plastic tube	0.176	0.990	1.975	2.966
	G-P Diff.[a]	−0.001[b]	−0.005[b]	−0.004[b]	0.009[b]
91	Glass tube	0.154	0.962	1.949	2.945
	Plastic tube	0.156	0.958	1.944	2.944
	G-P Diff.[a]	−0.002[b]	0.004[b]	0.005[b]	0.001[b]
182	Glass tube	0.135	0.930	1.926	2.927
	Plastic tube	0.135	0.933	1.921	2.952
	G-P Diff.[a]	0.000[b]	−0.003[b]	0.005[b]	−0.025[b]
364	Glass tube	0.111	0.910	1.891	2.896
	Plastic tube	0.107	0.915	1.880	2.900
	G-P Diff.[a]	0.004[b]	−0.005[b]	0.011[b]	−0.004[b]
Change 0–364 days	Glass tube	0.084[c]	0.106[c]	0.129[c]	0.123[c]
	Plastic tube	0.088[c]	0.101[c]	0.140[c]	0.119[c]
	G-P Diff.[a]	−0.004[b]	0.005[b]	−0.011[b]	0.004[b]

[a] Differences between means from 6 determinations on 3 glass and 3 plastic tubes.
[b] No statistically significant differences for blood stored in glass or plastic tubes.
[c] Statistically significant loss in ethanol content after 364 days of storage in glass and plastic tubes.

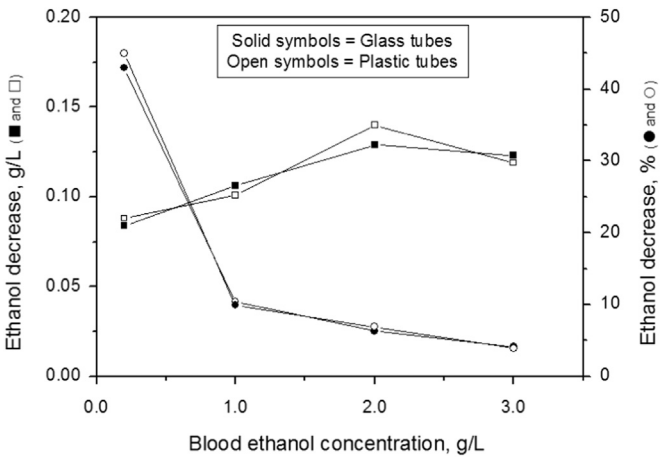

Fig. 1. Decreases in blood ethanol concentration in glass and plastic tubes after storage at 4 °C for 12 months. Both absolute decreases (squares) in concentration (g/L) and percentage decreases (circles) are plotted for the starting concentrations of 0.20 g/L, 1.0 g/L, 2.0 g/L and 3.0 g/L.

and 0.112 g/L (0.088–0.140 g/L) for plastic tubes at ethanol concentrations from 0.20–3.0 g/L. This meant that percentage decreases in ethanol content were highest (43–45%) at the lowest concentration (0.20 g/L) and 3.9–4.1% at the highest starting concentration of 3.00 g/L

Fig. 1 shows the trends in absolute and percentage decreases in ethanol content after 12 months of storage at 4 °C for blood kept in glass and plastic tubes. There were no analytically significant differences between tubes made of glass or plastic at all four starting concentrations of ethanol in blood from 0.20–3.0 g/L.

4. Discussion

The results of this study demonstrate that concentrations of ethanol in whole blood kept in glass and plastic evacuated tubes gradually decrease during storage in a refrigerator at 4 °C for 12 months. The losses of ethanol reached analytical significance after 14–28 days of storage, depending in part on the starting concentration. The absolute loss of ethanol did not seem to depend on the initial ethanol concentration over a range from 0.20–3.0 g/L. The mean decrease was ~0.11 g/L after 12 months storage. This meant that percentage losses of ethanol were greater for blood samples with lowest starting concentration of ethanol (see Fig. 1). The rate of ethanol disappearance from blood during storage was the same for specimens kept in glass or plastic evacuated tubes.

Several mechanisms have been proposed to account for losses of ethanol from blood samples during storage. The most plausible explanation is non-enzymatic oxidation of ethanol to acetaldehyde by oxyhaemoglobin present in fresh blood [10,11]. This oxidation reaction was prevented by use of sodium azide (0.5% w/v) as a preservative, which promoted the oxidation of oxyhaemoglobin to methaemoglobin [10]. Evaporation losses of ethanol during storage are less likely, because the blood tubes are only opened to remove aliquots for analysis. Moreover, at room temperature the blood/air partition coefficient of ethanol is strongly in favour of the liquid phase [12]. Appreciable losses of ethanol occur if the tubes of blood are left open for longer periods, as shown in a recent study [13]. A third explanation for lower analytical results when blood samples are analysed by HS-GC is a salting-out effect of ethanol caused by excess sodium fluoride in tubes with abnormally low volume of blood [14].

In some studies of ethanol stability greater losses occurred the more often the tubes were opened to remove aliquots for analysis [5]. Repeatedly opening the tubes introduces a new air-space above the blood sample, which probably facilitates further oxidation to acetaldehyde in the erythrocytes [11]. Several recent papers have investigated stability of ethanol in ante-mortem forensic blood samples during storage and all point towards a gradual decrease in concentration when sodium fluoride and oxalate were used as the preservatives [15,16]. Blood samples from subjects who had not consumed alcohol were negative initially (<0.0025 g/L) and remained negative when re-analysed after 13–39 months storage at room temperature [17]. In the same study the blood–ethanol concentration in drinking subjects (mean 0.64 g/L) decreased when the specimens were kept at room temperature or in a refrigerator at 4 °C.

The stability of ethanol in blood in glass or plastic tubes was compared in a small study from Norway [4]. After spiking hospital blood to give low (0.26 g/L) or high (4.48 g/L) concentrations of ethanol, the specimens were re-analysed after storage for 1 week, either at room temperature or frozen at −20 °C. Small decreases in blood–ethanol content were noted and the percentage loss was greatest at low starting concentration (0.26 g/L). No differences in ethanol loss were observed for blood kept in glass or plastic tubes.

The question of the amount of sodium fluoride necessary as a preservative for analysis of ethanol in ante-mortem blood samples has not been adequately investigated. One study showed that inclusion of fluoride ions made no difference when the tubes were refrigerated or kept at room temperature for 14 days before analysis [18]. This was confirmed in another study when the blood specimens were stored for 10 days [19]. The need for 100 mg NaF in ante-mortem forensic blood samples seems more of a historical tradition rather than a proven necessity.

Some forensic laboratories make use of 4–5 mL evacuated tubes for analysis of ethanol and other drugs of abuse in blood [20]. These tubes, originally intended for blood glucose analysis, contain smaller quantities of NaF, e.g. 10–30 mg in 5 ml tubes [19]. To the best of our knowledge, no direct comparisons of the amount of NaF necessary to stabilise blood ethanol content has been made [21]. Neither has it been established whether it makes any difference if heparin, EDTA or some other anticoagulant is used instead of potassium oxalate.

Important variables to consider in studies of ethanol stability are the temperature and duration of storage, type and amount of chemical preservatives, volume of air-space above the blood, and how often tubes are opened to remove aliquots for analysis. In one study loss of ethanol from opened tubes (mean 0.015 g/L) was greater than from unopened tubes (mean 0.010 g/L), after storage in a refrigerator for 13–39 months [17]. All bloods that were initially negative for ethanol content remained negative after short-term [22] and long-term storage under various conditions of time and temperature [15,17].

In conclusion, the present study confirms that ethanol concentrations in blood decrease gradually during storage of specimens at 4 °C for 12 months. The decrease reached analytical significance after 14–28 days of storage and was the same for specimens kept in glass or plastic tubes. The average decrease in blood–ethanol concentration after 12 months storage at 4 °C was ~0.11 g/L, and this did not seem to depend on starting concentration of ethanol or whether the evacuated tubes were made of glass or plastic.

Declaration

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