The Stability of Ethanol in Forensic Blood Samples with Storage Conditions

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Summary

The stability and variation with storage conditions of ethanol in forensic blood samples have been reviewed. Safe storage conditions for samples are outlined and suggestions made for research to be carried out into methods alternative to GLC for blood alcohol determinations.

Introduction

Alcohol (ethanol) when consumed moderately is a socially acceptable drug with generally accepted social benefits. However, alcohol is addictive and there are well recognised and widespread social disbenefits from its excess consumption. The chemico-legal aspects of alcohol intake and driving recognise the pathophysiology of alcohol which acts as a central nervous system depressant, with reduction of judgement and attenuation of psychomotor responses. Its consumption degrades the ability to drive carefully and react appropriately both to normal stimuli and unusual situations. As a result of the avoidable social costs, which in individual circumstances are some of the most tragic to afflict persons and families in the developed world, the law seeks to circumscribe drinking and driving. Thus, recognising the virtual impossibility of setting any systemic alcohol concentration at which it is safe to drive, it is an offence to drive, attempt to drive or be in charge of a motor vehicle when unfit to drive through drink. Separately, and for administrative convenience, it is an offence to drive, attempt to drive or be in charge of a motor vehicle if breath, blood or urine alcohol concentrations exceed prescribed limits. As the effects on the central nervous system depend on the concentration of alcohol delivered to the brain via the bloodstream the primary datum of interest is the blood alcohol concentration.

When, seeking to regulate drink-driving, blood alcohol testing was introduced in the UK a potential defendant in a criminal prosecution was afforded the statutory option to receive a portion of the blood alcohol sample so that their own independent analysis could be carried out (see RSC https://www.rsc.org/globalassets/13-help-legal/help/road-traffic-analysts-booklet.pdf). Small and decreasing numbers of defendants avail themselves of this option and when they do the chain of custody of their sample may not be as well defined as that of the prosecution sample. Thus, the stability of blood samples assumes considerable importance when conflicting evidence of their ethanol contents is adduced in court. It is unlikely that both samples have been stored identically or their alcohol content measured simultaneously in time

and these aspects will come under intense forensic scrutiny. Here we review studies of the stability of blood samples under various storage conditions and, in particular, the stability of their ethanol content.

Review of the Literature on the Stabilisation of Blood Prior to Analysis

a   For Clinical Purposes

Studies of the preservation of blood samples for use in clinical analyses commenced in the early twentieth century, at first with limited success. In 1920, Ambard reported that a mixture of 1 part NaF and 2 parts KH_2PO_4 at 15g/l prevented glycolysis of blood for at least 6 hours[1]. The use of sodium fluoride was examined several times in the 1920's[2-6] for the preservation of blood sugar for up to 6 days[2]; non-protein nitrogen compounds up to 2 weeks[3, 4]; cholesterol, in addition to non-protein nitrogen compounds[5, 6] for sterile samples. The addition of thymol was found to be advantageous with non-sterile samples[5].

b   For Forensic Blood Alcohol Determinations

By the mid-century with the increase in road vehicular traffic there was a significant interest in the determination of alcohol in motor vehicle drivers' blood and in the stability of samples during the period between sampling and analysis. Several authors reported that sodium fluoride satisfactorily preserved ethanol in refrigerated blood[7-9].

Glendening and Waugh studied in detail the effects of different storage conditions[10]. They found no significant changes using 100 mg NaF in each sample as preservative when stored at room temperature up to 2 months, or deep frozen up to 9 months using Leifert's[11] modification of Kingley and Current's spectrophotometric determination after volatilisation of ethanol into acidified potassium dichromate solution[12]. In their literature review it was noted what appears to be the first application of sodium fluoride to stabilise blood samples prior to analysis for ethanol, that by Mewbarr and Myers for autopsy samples, stored in screw-capped bottles, in 1954[13], who stated,

> *"Sodium fluoride will satisfactorily preserve blood for at least two weeks without refrigeration. In our experience blood alcohol determinations on fluoridated blood under these conditions have shown a drop in the alcohol level of less than one point in the second decimal place".*

The stability of ethanol in stored blood became important in the UK following introduction of the Road Safety Act, 1967. This Act made it an offense for a motor vehicle driver to have an alcohol concentration in excess of 80 mg per 100ml in their blood. In a number of cases discrepancies were found between the forensic science laboratory results and those of the defence analysts. A common feature in these cases was the relatively long period of storage of the defence sample at room temperature prior to analysis. At the time it was considered that

the available data was inadequate to interpret such discrepancies when they occurred[14]. Five factors, time, fluoride concentration, ethanol concentration, temperature and type of container were studied by gas chromatography[15] in a factorial experiment. The three significant factors, temperature, presence of fluoride and time of storage were studied in detail. The rate of loss of ethanol was found to be due to the growth of micro-organisms inhibited by fluoride at concentrations above 0.5% w/w, strongly dependent upon temperature over the range -20 to 62ºC (effectively stable at -20ºC, losses increasing from .002 to 6.0 mg% per day from 4-37ºC) and time of storage (decreasing by 2.5% after 13 days and 8.1% after 35 days at room temperature). The oxidation mechanism not inhibited by fluoride was proposed to be due to an oxyhaemoglobin intermediate[16].

Since this pioneering UK research numerous others have examined aspects of the problem. In 1987 Hayden *et al* reported, by GLC, an average decrease in alcohol in blood (stabilised with sodium fluoride and potassium oxalate) on storage of 2.4 mg/100ml at 4ºC over 30-50 days[17]. Meyer *et al* in an extensive study using ADH and by GLC confirmed the earlier results of Brown *et al*[18], as did Winek and Paul for short term storage (up to 14 days) at 0-3ºC and at 22-29ºC[19]. In a long-term study, 3- and 6.75-years' storage of unopened tubes at room temperature in the presence of fluoride Chang *et al* found losses of 20-40 mg%[20]. Shajani *et al* studied the stability of fluoride-stabilised forensic blood samples by GLC after storage at 4ºC for 39 weeks and at room temperature for 18 weeks and found no essential change in ethanol contents[21]. Winek *et al* using serum samples spiked with ethanol, stored at 26.7, 32.2 or 37.8ºC, with or without preservative were stable up to 35 days whereas the blood samples submitted by external agencies showed significant losses, in the range 15-19%, attributed to chemical oxidation[22].

In a statistically rigorous inter-laboratory study Dubowski *et al* commented that many of the prior studies contained contradictory results[23]. In order to address the question, *"how long survey-validated blood-alcohol specimens could be used appropriately as analysis controls"*, and also to assess results for delayed analysis they had prepared sterile, sealed sets of pooled whole blood containing ethanol at 90, 150 and 230 mg/100 ml with EDTA and heparin anti-coagulants and fluoride preservative in all specimens. Subsets contained, in addition, either sodium azide as an additional preservative or carbon monoxide. The carbon monoxide was added to reduce the available oxy-haemoglobin to lessen the oxidation of ethanol. The experimental design did not provide for the separate evaluation of the CO treatment, found previously to stabilise samples. The samples were stored by the manufacturer at 2-8ºC until shipped at ambient temperature and stored at 4ºC thereafter, separate vials were analysed monthly by six laboratories. It was found that azide-containing samples showed no loss during one year of storage, azide-free samples showed minimal loss in the first four months of storage and less than 5% over one year.

Ferrari *et al* found the ethanol concentration decay with time showed pseudo-first-order kinetics, in confirmation of Smalldon and Brown's[16] earlier findings, but failed to reference them[25]. Kristoffersen *et al* also confirmed Smalldon and Brown's conclusion that the decrease in blood alcohol concentration with time is due to oxidation by red blood cells resulting in a nearly equivalent increase in acetaldehyde concentration[25]. In 2007 Jones stressed the

importance of using the appropriate standard deviation when searching for changes in BAC during storage since imprecision tends to change as a function of concentration and when reporting results in court for stored samples, samples with 1% sodium fluoride were stable for at least 6.5 months stored at 4ºC[26].

Mandic-Radic *et al* studied the stability of BAC in stabilised samples using a commercial ADH kit, after storage for 7 and 14 days at room temperature the losses were 7.9% and 22.4% respectively, at 4ºC the loss was 4.2%, after 3 months, at -20ºC samples were stable for 12 months[27].

Penetar *et al* in a BAC in plasma, serum, whole blood ratio study, examined by GLC separate sets of blood samples, on the day collected, after storage at room temperature for 1 and 10 days, and after 10 days of refrigerated storage. They reported that the type of storage tube or the type of additive did not affect the stability; storage time and temperature are the main variables. Samples stored at 4ºC were stable for at least 10 days[28].

Shan *et al* examined the stability of retained opened and unopened samples from drink driving forensic samples after refrigerated storage for 13-39 months. The mean loss from unopened tubes was 0.010 g/dL[29]. Storage at 38ºC for one week caused a small loss in BAC, but within the accuracy of the method.

Tisicone *et al* using forensic samples noted significant loss based on blood volume within a tube, when tubes had been reopened after prior analysis and with time of storage at room temperature[30]. Kocak *et al* used ADH to measure BAC in fluoride-stabilised samples and reported samples were stable at -20ºC for up to 3-4 months provided that the tubes were nearly completely filled[31], as noted earlier by Tisicone *et al* in their long storage time study of forensic case samples[30] as did Stojilkovic *et al* for sample storage at -18ºC, for up to 180 days[32].

Vance *et al* give the results for the analysis of multiple vials of preservative-treated whole blood samples, from ethanol-positive and ethanol-negative volunteers, over 0-60 days' storage, at 3-20ºC[33]. For the alcohol-positive subjects the group means after 56 days refrigerated and 60 days at room temperature were within the estimated uncertainty of the GLC method. Jones and Ericsson report that BAC gradually decreases with time during storage at 4ºC over 12 months and were analytically significant after 12-28 days independent of their being stored in glass or plastic tubes[34].

Zamengo *et al* have developed a software application to assist forensic analysts with the challenging role of assessing measurement uncertainties in assessing compliance or otherwise of BAC results with the legal limits in force[35]. Laurens *et al,* after paying particular care over measurement uncertainty, found using GLC-MS that spiked samples stored at 4ºC, with fluoride present, were stable with time, but not at room temperature[36].

In a large inter-laboratory study carried out in Italy all participants used head-space GLC on two 4 mL blood samples provided in 5 mL tubes. The 4 mL blood volume was selected to avoid possible cracking of tubes during freezing and thawing of the contents. The samples were

delivered to participants in expanded polystyrene boxes loaded with 2 ice bricks. Forty per cent of the laboratories kept samples frozen at -20ºC whereas the others used refrigeration at 4ºC. There were no statistically significant differences between BAC in frozen or refrigerated samples over 20 days[37].

Alternative Methods to GLC for the Determination of Ethanol in Blood

The earlier publications on BAC mainly used one of two methods, spectrophotometry following distillation or diffusion in an oxidising solution or enzymatically using ADH. Recently some interesting non-destructive alternatives have been put forward. Shama *et al* examined the use of horizontal attenuated total reflectance-Fourier transform infrared spectroscopy to determine BAC in whole blood, using the asymmetric stretching frequency of the C-C-O group of ethanol in water at 1,045 cm^{-1} [38] based on their prior study of the determination of methanol and ethanol in alcoholic beverages[39]. The Beer's law plot of BAC was linear over the range 24-790 mg/100 mL, (r^2=0.999; sd=0.0023) and results agreed with those by GLC to within ±1 mg/100 mL. The method is rapid, simple and non-destructive of the sample. FTIR has been shown to distinguish between the bloods of alcoholic and non-alcoholic patients[40].

Bock's analysis of blood serum by high-field NMR demonstrates that it is possible to detect a wide range of compounds of clinical interest[41]. This was followed by a quantitative study by Pappas *et al* of ethanol in human blood serum. Calibration plots were linear from 0-1490 mg/L with a detection limit of 15 mg/L[42]. Davin *et al* showed excellent correlation of ethanol concentrations in plasma when determined enzymatically and by NMR[43]. Zailer and Diehl have produced a validated NMR method for BAC using either whole blood or serum[44]. For most of the study whole blood samples were used to gain time and use fewer working steps. A drop of blood, 20μL, was found to be adequate which could lead to a simplification of sample collection. Forensic case samples were additionally determined by GLC and using ADH and the results correlated excellently with those by NMR. Due to using a non-destructive method follow-up determination of DNA is possible for use in resolving legal disputes.

Comparison of Prosecution Analytical Data with that of the Defence in BAC Forensic Cases

When the results of the prosecution (mean=x_1) and defence (mean=x_2) do not agree these should be tested by the "null hypothesis" to determine statistically if the difference between the results is different from zero, using Student's *t* test[45].

As the two sets of data will have been produced under similar well-regulated conditions it can be assumed they will have very similar coefficients of variation (CV) or relative standard deviation (RSD), thus the individual standard deviations (s_1 and s_2) can be used to calculate a pooled standard deviation to use in the *t* test.

$$CV = 100s/x$$

$$s^2 = \{(n_1 - 1)s_1{}^2 + (n_2 - 1)s_2{}^2\}/(n_1 + n_2 - 2)$$
where n_1 and n_2 are the number of determinations in the two sets of results

$$t = (x_1 - x_2)/s \ \sqrt{(1/n_1 + 1/n_2)}$$

If the experimental value of t is greater than the critical value for 2 degrees of freedom (DF= n_1+n_2-2) in the tables for *t*, the null hypothesis is rejected and the difference between the two sets of results is significant at the appropriate confidence level.

By way of example, in a recent case, the prosecution reported "BAC not less than 82mg/100 mL", the defence reported "BAC not less than 78mg/100 mL" for the sample that had been stored refrigerated by the defendant prior to analysis.

Assuming that:
- The coefficients of variation are the same for both sets of data at 2.5%

- The results were each based on two aliquots of the sample and duplicate injections were run on two analytical columns with different stationary phases, that is 4 data points in each data set. To allow for any column effects the data is treated as two determinations, ie one degree of freedom each.

Using the equations above, the standard deviation for the prosecution data is 2.05mg/100mL and that of the defence is 1.95mg/100mL. The pooled estimate of the standard deviation is 2.00mg/100mL and the experimental value *t*=2.00. The critical tabulated value of *t* for 2 degrees of freedom is 4.3 at the 95% confidence limit. This is more than the experimental value hence the null thesis is accepted. The results 82mg/100mL and 78mg/100 mL do not differ statistically at the 95% confidence limit. The court thus accepted the defence case that their sample did not exceed the legal limit of 80mg/100 mL and found for the defence. The pooled mean of all the results is 80 mg/100mL, ie not greater than the limit of 80 mg/100 mL.

Conclusions

a   Advice with Regard to the Defence Sample of Whole Blood

The data in the literature reviewed confirm the earlier conclusions of Brown *et al*[14] that the ethanol content of whole blood samples with fluoride as a preservative remain constant within their experimental determination by GLC when stored under refrigeration for one month or for one year if frozen at -20°C. Thus, defence samples should soon after receipt be stored under refrigeration or preferably in a deep freeze (with care that the glass tube does not crack).

Further, in order to best preserve the forensic integrity of a defence sample, it is recommended that the defendant's whole blood sample be stored in an independent refrigerator in order to provide unbiased evidence of its storage prior to transfer to the analytical chemist specified in the certificate presented to the court.

b   Suggestion for Future Studies

A series of international inter-laboratory studies are clearly desirable as is a universally agreed operating procedure for the determination of BAC. The examination of small whole blood samples by high field NMR is worthy of more detailed study in view of the reduction in time and number of working steps in addition to the wide range of compounds of clinical interest it is possible to determine simultaneously to the ethanol content. In particular, illegal drugs and their metabolites should be examined, as has been done by NMR for gamma-hydroxybutyrate in oral fluids[46].

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