Blood Analysis by Headspace Gas Chromatography: Does a deficient sample volume distort ethanol concentration?

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ABSTRACT

This study was prompted by a recent judgment in the Royal Courts of Justice (*Gregory* v. *Director of Public Prosecutions*, 2002) in a case of driving a motor vehicle after consuming too much alcohol (Road Traffic Act 1988). An expert witness for the defence alleged that a deficient volume of blood in the tube sent for analysis meant an excess amount of sodium fluoride (NaF) preservative, which would increase the concentration of ethanol, determined by headspace gas chromatography (HS-GC), owing to a salting-out effect. The prosecution did not produce expert evidence to rebut this argument and the drunk driving suspect was acquitted.

A small volume of blood and excess sodium fluoride might have increased the concentration of ethanol in the air-space in the tube sent for analysis but this does not mean that the result of the HS-GC analysis would be higher. This follows because prior to analysis an aliquot of blood is removed and diluted (~10 times) with n-propanol as the internal standard. The dilution lowers the concentration of NaF in the blood and for quantitative analysis the ratio of the ethanol to n-propanol response is measured. The use of a ratio also helps to compensate for any salting-out effect of ethanol. Our experiments showed that a deficient volume of blood and excess NaF actually lowered the concentration of ethanol by 2-3% compared with heparinised blood. Seemingly, n-propanol (n-PrOH) a 3-carbon straight chain alcohol is salted out slightly more effectively than the 2-carbon ethanol (EtOH) causing a lower peak area ratio (EtOH/n-PrOH) and a lower apparent concentration of ethanol. In a separate study, we showed that the concentration of ethanol was lowered even more when a 4-carbon alcohol (t-butanol) was used as the internal standard.

INTRODUCTION

Headspace gas chromatography (HS-GC) is widely used in forensic science and toxicology laboratories for analysis of ethanol as well as other low molecular volatiles in biological specimens (Machata, 1967; Tagliaro et al., 1992; Seto, 1994). This analytical technique entails sampling the air-space, called the headspace, above the liquid specimen (e.g. blood or urine) after equilibrium is reached in an air-tight glass vial maintained at a constant temperature of 50 or 60°C (Snow and Slack, 2002). The concentration of a volatile substance in the headspace vapour at equilibrium is proportional to the concentration in the liquid phase as predicted from the liquid/air partition coefficient for that substance and the temperature of the system (Kolb and Ettre, 1997).

The theory of HS-GC is well established and several books are available on the subject (Hachenberg and Schmidt, 1977; Kolb, 1978; Ioffe and Vitenberg, 1984; Kolb and Ettre, 1997). In brief, when a dilute solution of a volatile substance is allowed to equilibrate in a closed container at constant temperature the following concentration relationship holds after reaching equilibrium:

$$\frac{\text{Concentration of substance in the liquid phase } (C_L)}{\text{Concentration of substance in the air phase } (C_A)} = \text{Constant } (k_{L/A})$$

The concentration of ethanol in the air-phase is proportional to the peak area response on the gas chromatogram. The partition constant ($k_{L/V}$) does not need to be known and is assumed to be the same for the aqueous

ethanol calibrator and the blood sample after an appropriate dilution with internal standard (Dubowski, 1975; Vitenberg, 1991). Because the concentration of ethanol in the aqueous standard (C_L) is known and the concentration in the air-space is obtained by analysis, it becomes a simple matter to calculate the concentration of ethanol in the blood samples. A calibration curve is constructed covering the concentration range of interest (e.g. 10-500 mg/100 mL) with aqueous ethanol standards obtained from a traceable source. The ratio of the ethanol response to the internal standard response is plotted as the y-variate and the concentration of ethanol in the standard as the x-variate to give a calibration plot (Dubowski, 1975). The concentration of ethanol in the blood specimen is derived from the linear relationship between peak area ratio and concentration in the standards by least squares regression analysis (Sutheimer et al., 1969).

In most countries the blood specimens for forensic analysis of ethanol are taken from a cubital vein with the aid of evacuated tubes (5-10 mL) called Vacutainer tubes. These tubes contain a fixed amount of anticoagulant, e.g. potassium oxalate or heparin and a preservative (enzyme inhibitor), such as sodium fluoride, usually 100 mg in a 10 mL Vacutainer tube. If the tube sent for analysis is only partially filled with blood this means that the concentration of NaF will be higher than expected. However, according to a standard reference book (The Merck Index, 12th edition), the solubility of NaF in water at 20°C is only 4.0 g/100 mL (4%). Accordingly, if the volume of blood in a 10 mL tube is less then 2.5 mL the solution will be saturated with NaF (0.1/2.5 = 4%).

In this study we evaluated whether a deficient volume of blood or urine, and therefore an excess amount of NaF, caused the concentration of ethanol determined by headspace gas chromatography to be higher than expected. We evaluated the use of both n-propanol and t-butanol as internal standards and ethanol was determined in blood, water and urine.

MATERIALS AND METHODS

Preparation of specimens

Specimens of blood and urine were collected from the same male volunteer who had abstained from drinking alcohol for several days. These biological specimens were spiked with ethanol (10% w/v) to give target concentrations close to 100 mg/100 mL. An aqueous solution of ethanol was made to contain the same concentration.

Different volumes of blood, urine and water were introduced into 10 mL glass tubes with conical bottoms and screw-on caps. In another series of experiments, 25 mL glass bottles with screw-on caps were used. The same amount of sodium fluoride (100 mg) was weighed into each 10 mL tube before adding different volumes of whole blood, urine or water containing ~100 mg/100 mL ethanol. The volumes of the liquids added to the tubes and the expected concentrations of NaF (% w/v) were; 0.5 mL (20%), 0.75 mL (15%), 1.0 mL (10%), 2.0 mL (5%), 4.0 mL (2.5%), 10 mL (1%). One tube did not contain any fluoride additive and served as a control and heparin was used to prevent coagulation of the blood sample. The salt and liquid samples were thoroughly mixed on a Rotamix and kept in air-tight tubes overnight at room temperature. The tubes were mixed again the next day before aliquots (100 μL) were removed for analysis by HS-GC.

In another study, different amounts of NaF were weighed into screw-capped flasks (25 mL) before adding the same volume of urine (10 mL) to each. The contents of the flasks were mixed on a Rotamix and left at room temperature overnight, then mixed again the next morning before aliquots (100 μL) were removed for analysis by headspace gas chromatography. It was obvious that the flasks containing most NaF were saturated.

Headspace gas chromatography

Prior to gas chromatographic analysis all specimens including the calibrators were diluted 1 + 10 with a Hamilton diluter dispenser instrument whereby 100 μL of water, urine or blood-ethanol were diluted with 1 mL aqueous n-propanol (8 mg/100 ml) as the internal standard. In another series of experiments with

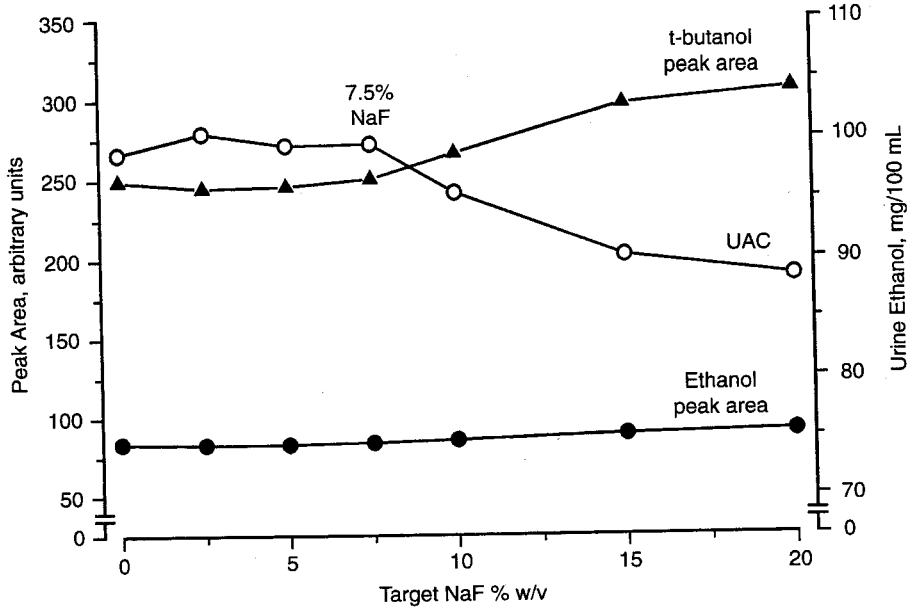

Figure 1. Relationship between the concentrations of ethanol in urine (UAC) containing different amounts of sodium fluoride as determined by headspace gas chromatography. Prior to analysis the urine was diluted (1:10) with t-butanol as an internal standard.

urine, t-butanol (5 mg/100 mL) was used as the internal standard. The analysis was done with HS-GC equipment from Perkin-Elmer and all measurements were carried out in duplicate. The peak areas of the ethanol and n-propanol response on the chromatograms were noted and used for quantitative analysis.

RESULTS

Figure 1 shows the concentration of ethanol determined in urine in relation to the concentration of NaF in 25 mL sample bottles. The ethanol concentration seemed to remain constant up until 7.5% w/v NaF decreasing thereafter as the concentration of NaF increased to 20% w/v. With high concentrations of NaF in solution, the peak area of the t-butanol response increased more than the ethanol response and this data is summarized in Table 1. This shows mean SD and CV% for

Table I. Effect of different amounts of sodium fluoride on the concentration of ethanol determined in urine (UAC) by HS-GC with t-butanol as the internal standard. Mean values (N = 5 flasks), standard deviation (SD) and coefficient of variation (CV%) of the peak areas of ethanol (EtOH), t-butanol (t-BuOH) and the ethanol/t-butanol ratio (E/t-B) are shown.

Statistic	Sodium fluoride 0% w/v,			Sodium fluoride 10% w/v[1]			Sodium fluoride 20% w/v[1]		
	EtOH	t-BuOH	E/t-B	EtOH	t-BuOH	E/t-B	EtOH	t-BuOH	E/t-B
Mean	83.7	249.1	0.336	87.7	277.0	0.316	90.6	311.2	0.291
SD	1.28	4.1	0.020	1.53	8.7	0.053	0.63	13.7	0.012
CV%	1.5	1.7	0.61	1.8	3.1	1.68	0.69	4.4	4.2
UAC	98.9 mg/100 mL			93.9 mg/100 mL			86.8 mg/100 mL		

[1] Saturated with NaF.

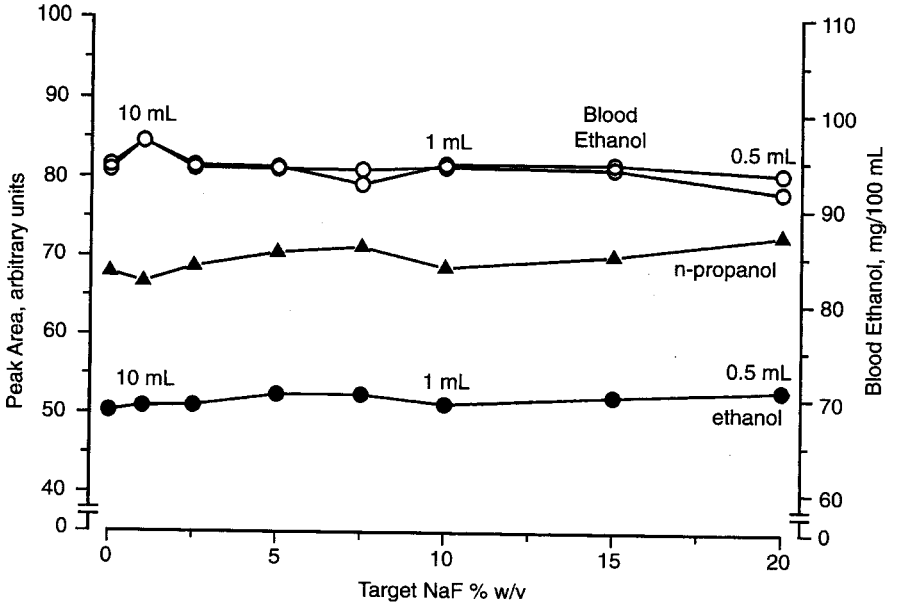

Figure 2. Relationship between ethanol concentration and volume of blood in the tubes containing 100 mg sodium fluoride. Prior to analysis by headspace gas chromatography aliquots of blood (100 μL) were removed and diluted (1:10) with n-propanol as internal standard.

peak areas of ethanol (E), t-butanol (t-B) and the E/t-B ratio as well as the concentrations of ethanol in the urine according to HS-GC analysis. The concentration of ethanol without any NaF was 98.9 mg/100 mL and this decreased on average by 5 mg/100 mL (5%) and 12 mg/100 mL (12%) when the nominal concentrations of NaF were 10% w/v and 20% w/v, respectively. These are reported as nominal concentrations because the solutions were saturated with salt. Results in Table 1 show that the lower ethanol concentrations are caused by decreasing E/t-B ratios at high salt concentration. For specimens of urine containing 10% w/v and 20% w/v NaF, the peak areas for ethanol increased by 3% and 12% respectively whereas t-butanol increased by 9% and 19% respectively.

Figure 2 shows the relationship between blood-ethanol concentration and the nominal concentration of NaF in tubes with blood volumes ranging from 10 mL to 0.5 mL.

Table II. Effect of deficient volume of sample and excess sodium fluoride on the concentration of ethanol in blood, urine and water determined by headspace gas chromatography. The volume of sample was 0.5 mL contained in a 12 mL volume glass tube with 100 mg sodium fluoride.

Liquid phase	EtOH concentration without NaF	EtOH concentration with 20% w/v NaF[1]	Percentage decrease in EtOH with excess NaF
Water-ethanol	99.9 mg/100 mL	97.6 mg/100 mL	2.4%
Urine-ethanol	99.9 mg/100 mL	95.5 mg/100 mL	3.5%
Blood-ethanol	94.3 mg/100 mL[2]	92.7 mg/100 mL	1.7%

[1] Nominal concentration because the solutions were saturated.

[2] Control blood specimen containing heparin as anticoagulant.

Despite the wide variation in blood volumes the concentration of ethanol decreased by only 2-3% (Table II). The peak areas for ethanol and n-propanol increased only slightly when the concentration of NaF increased as the volume of blood decreased.

DISCUSSION

Procedures and conditions for HS-GC analysis of volatiles differ in different laboratories in terms of the sample pre-treatment used, the chromatographic conditions, such as oven, injector and equilibrium temperatures, as well as the stationary phase used, and whether a traditional packed column, capillary or wide bore column was fitted to the GC (Sutheimer et al., 1969; Dubowski, 1975; Anthony et al., 1980; Jones and Schuberth, 1989; O'Neill et al., 1999). In most forensic laboratories the blood specimens are diluted 1:10 or 1:5 with an internal standard consisting of an aqueous solution of another alcohol e.g. n-propanol or t-butanol (Christmore et al., 1994).

An important requirement for quantitative HS-GC analysis when aqueous calibrators are used is compensation for differences in the matrix compared with the biological sample (Strassnig and Lankmayr, 1999). This is necessary so that the same liquid/air partition coefficients apply to both standard and biological specimens (Watts and McDonald, 1987) and this can be achieved by 10-fold dilution of blood with internal standard. Note that whole blood is ~85 % w/v water so the matrix problem is effectively eliminated (Machata, 1967; Sutheimer et al., 1969). The use of an internal standard helps to compensate for any fluctuations in operating conditions that might arise during an analytical run because the peak height or peak area ratios (ethanol-to-internal standard) are measured and used for quantitative analysis. Other ways to eliminate matrix effects include saturating the aqueous standard and the blood sample with an inorganic salt such as potassium carbonate or sodium sulphate (Dubowski, 1977; Christmore et al., 1984; Watts and McDonald, 1990).

The Vacutainer tubes used to draw blood for forensic analysis of ethanol (gray-stoppered 10 mL tubes) contain 100 mg sodium fluoride as an enzyme inhibitor and 22 mg potassium oxalate as an anticoagulant. Owing to difficulties in sampling blood from uncooperative subjects or when a superficial vein is hard to discern, the volume of the blood specimen submitted for analysis can vary widely. In extreme cases the volume of blood might be as little as 0.5 mL or as much as 10 mL, a 20-fold difference. Good laboratory practice requires that the volume of the blood samples received be noted in every single case. With an abnormally small volume of blood, because of the low solubility of NaF in water and presumably also in blood, the specimen is likely to be saturated if it is less then 2.5 mL. Such a small volume as 0.5 mL in a 10 mL Vacutainer tube represents an extremely deficient sample, making it troublesome to withdraw the 100 µL aliquots needed for analysis in a reproducible way – a fact that might impact on the overall precision and accuracy of the assay.

The salting-out of nonelectrolytes from aqueous solution is well studied and depends on the attraction of water molecules for the Na^+ and F^- ions in solution (Long and McDevitt, 1952). The effect of excess salt raises the vapour pressure of non-electrolytes such as ethanol, n-propanol or t-butanol, causing the concentrations in the headspace to increase. Studies have shown that alcohols like n-propanol (3-carbons) are salted out more effectively than ethanol (2-carbons), resulting in a smaller ethanol:n-propanol peak area ratio and accordingly lower apparent concentrations of ethanol (Watts and McDonald, 1987; Watts and McDonald, 1990). The salting-out effect of t-butanol (4-carbons) was seemingly greater than that for n-propanol, which meant that the apparent concentration of ethanol in urine was even lower then expected (Table I).

An equilibration study with solutions of ethanol in water showed that 1% NaF raised the concentration of ethanol in the air-space by about 8% compared with no NaF present (Jones, 1983). But ethanol was measured directly in the air-space without any dilution with internal standard because there was no interest in measuring the liquid phase

concentration of ethanol. If a blood specimen contained 4% NaF this becomes 0.36% NaF after diluting 1 + 10 (11 times) with aqueous n-propanol and the salting-out effect of this low concentration is negligible. Furthermore, both ethanol and n-propanol would be salted-out and when the ratio of peak areas (EtOH/n-PrOH) is measured this compensates for any effect of salt on the ethanol peak.

In an extreme situation with an unusually small volume of blood in the sampling tube (0.5 mL) and a large volume of headspace (10 mL), corresponding to a liquid/air phase ratio of 1:20, some might wonder whether volatiles are lost into the air-phase during the transport and storage of the tubes. If this happened, it would lead to a lower concentration in the blood specimen when this was analysed by HS-GC. However, it is important to note that the liquid-air partition coefficients of ethanol in blood, water and urine at room temperature are strongly in favour of the liquid (~5000-6000:1) so there is no appreciable loss into the air-space despite very small volumes in the tube (Harger et al., 1950; Jones, 1983). For other volatiles, such as acetone, chloroform, ether or toluene, the situation is different because these substances have much smaller liquid/air partition ratios compared with ethanol (Sato and Nakajima, 1979).

Some work has already been done to test the influence of the volume of the blood specimen in the sampling tube on the results of HS-GC analysis of ethanol (Solanky, 1994; Jones, 1994). These short communications (letters to the Editor) showed that with an abnormally low volume of blood there was a slight decrease in the concentration of ethanol determined by HS-GC. The present study, which was prompted by the recent Appeals court decision in the case of *Gregory* v. *Director of Public Prosecutions*, expands on and confirms these earlier findings.

In conclusion, this study showed that an unusually small volume of blood in the tubes sent for analysis of ethanol and an excess amount of NaF caused a slight lowering (by 2-3%) of the apparent concentration of ethanol determined by HS-GC compared with blood without NaF. The explanation appears to be a preferential salting-out of internal standard (n-propanol and t-butanol) compared with the 2-carbon ethanol making the peak area ratios (ethanol/internal standard) smaller, resulting in a lower apparent concentration of ethanol as determined from the calibration curve. When a deficient volume of blood or urine is sent for determination of ethanol by HS-GC, the drunk driver gains a slight advantage compared with a tube filled with blood and no excess NaF.

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STATUTES

Road Traffic Act, 1988