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Research Paper

Pre-analytical factors related to the stability of ethanol concentration during storage of ante-mortem blood alcohol specimens

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ABSTRACT

Sterile ante-mortem blood specimens were spiked with ethanol at the South African blood alcohol legal concentration limits of 0.20 g/L and 0.50 g/L and were stored in tubes containing sodium fluoride over a period of twenty-nine weeks under refrigeration (4 °C) and at room temperature (22 °C) to study the stability of the ethanol concentrations over time. Those stored under refrigeration were found to be stable, while a significant decrease in ethanol concentration at 99% confidence was observed in those stored at room temperature.

Additional blood specimens, also spiked with ethanol, were inoculated with the dimorphic fungus *Candida albicans* at five different levels (1×10^6 cells/mL, 5×10^5 cells/mL, 1×10^4 cells/mL, 5×10^3 cells/mL and 5×10^1 cells/mL) and stored with and without sodium fluoride at 4 °C and 22 °C. The ethanol concentrations were monitored for nine weeks unless no fungal colonies were detected. Regardless of the presence or absence of NaF in samples – sterile or otherwise – storing specimens at 4 °C was sufficient to maintain the integrity of blood alcohol concentrations.

The ethanol analyses were performed with an in-house validated isotope dilution gas chromatography-mass spectrometry analytical method on newly opened specimens once a week after which significance testing was performed to draw conclusions regarding changes in ethanol concentrations with measurement uncertainty in mind.

1. Introduction

Blood alcohol analysis is one of the most often requested forensic tests in South Africa and is mostly used for law enforcement purposes. The legal limits in South Africa are 0.20 g/L and 0.50 g/L for professional drivers and public drivers respectively.[1] The standard protocol requires that a qualified phlebotomist draws blood from an antecubital vein of a driver within 2 h after arrest. Blood is collected directly into a sterile evacuated tube containing potassium oxalate as anticoagulant and sodium fluoride (NaF) as inhibitor.[2,3]

Protocol prescribes that the specimens be kept cool for the period of transportation to the laboratory, and thereafter be stored under refrigeration at 4 °C until analysis.[4] The sodium fluoride concentration should be above 1% after filling the tube with venous blood, and this concentration is routinely determined and reported as part of the blood alcohol test report.[3]

The reliability of blood alcohol test results is being questioned by defence teams due to claims in literature that the alcohol concentration may increase with time, as reported by Chang et al. and Blume et al. on post-mortem blood.[5,6] The relevance of studies making use of post-mortem blood attempting to explain changes in ante-mortem blood ethanol concentrations is questionable. In the presented study, ante-mortem blood was used. Yajima et al.[7] showed that the presence of *C. albicans* caused an increase in ethanol concentration, provided glucose was added, and that the ethanol production was proportional to the glucose concentration. It should be noted that no ethanol increase was observed in specimens that did not have added glucose.

Little emphasis however is placed on the fact that the majority of studies found in literature make use of either blood obtained from a blood bank, containing dextrose,[5] an additional substrate for microbial growth, or post-mortem blood, where the blood biochemistry has changed.[8] Neither of these adequately simulates the ante-mortem blood specimens routinely obtained for blood alcohol analysis. Nevertheless, the reasons for the changes in alcohol concentration are cited in literature as microbial contamination of blood specimens[5,6,9] and non-enzymatic oxidation.[10–12] Possible microbial contaminants capable of

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producing ethanol include *Candida albicans*, *Proteus* sp., *Escherichia coli*, *Staphylococcus* sp.[13] However, it has been shown that contamination with micro-organisms from post-mortem specimens introduced into ante-mortem specimens by diluters or pipettes can also cause a substantial decrease in blood alcohol concentration of the ante-mortem specimen.[4]

The stability of blood alcohol concentrations in blood specimens is of paramount importance. Should the ethanol concentration decrease it would only be to the benefit of the defendant and justice may not be served, while if it were to increase they might be prosecuted unfairly. It is thus imperative for a reliable and accurate blood alcohol result that the ethanol concentration remain unchanged from sampling to analysis – that is, with no *significant* increases or decreases.[14,15]

Fluoride acts as a potent inhibitor of enolase, one of the enzymes in the glycolysis pathway, whereby a micro-organism like *Candida albicans* can convert blood glucose into ethanol anaerobically.[16] It should be noted, however, that according to literature the absence of preservative (NaF) in contaminated blood specimens causes the whole blood glucose level to decrease more rapidly, thereby removing the major substrate of ethanolic fermentation.[17]

It should also be considered that ethanol is a volatile substance. As such it is possible that evaporation may cause a decrease in ethanol concentration.

In order to ensure the validity of test results, testing laboratories require procedures for safe handling, transport and storage of samples and reference materials in order to protect specimen integrity. This inherently necessitates the laboratory to prove analyte stability over the period of storage. Where there is a backlog of specimens in a laboratory this becomes even more critical. Pre-analytical studies simulating the storage conditions must therefore be conducted to assess the impact of storage on the final result.

In this study, an in-house validated gas chromatography – mass spectrometry isotope dilution analytical procedure was used to obtain information on the stability of ethanol concentration in ante-mortem blood specimens inoculated at different concentrations of *C. albicans*. In addition to being stored at either room temperature or under refrigeration, some specimens contained sodium fluoride (NaF) while others did not. The expanded measurement uncertainty of the analytical method was used to gauge the significance of any changes in blood alcohol concentration.

2. Materials and methods

2.1. Reagents and materials

A certified ethanol reference standard (200 g/L) was obtained from the National Metrology Institute of South Africa (NMISA) and stable isotope labelled ethanol-D6 (99%) was obtained from Sigma Aldrich, Midrand, South Africa.

Sodium hydrogen carbonate (99%) and sodium carbonate (99%) were purchased from Merck, Steinheim, Germany; pentafluorobenzoyl chloride (99%) (PFBCl) was obtained from Sigma-Aldrich, Midrand, South Africa; sodium hydroxide pellets (97.0%) were acquired from Merck, Worli, Mumbai.

All solvents were analytical grade and were used without further preparation.

Dichloromethane (pesticide grade) and isopropanol (99.9%) were obtained from Sigma-Aldrich, Steinheim Germany. Acetonitrile (HPLC grade) was purchased from Sigma-Aldrich, Midrand, South Africa, while deionised water was sourced from Merck, Modderfontein, South Africa.

A *Candida albicans* ATCC 90028 strain was obtained from the Department of Medical Microbiology, Tshwane Academic Division (TAD), National Health Laboratory Service (NHLS). Phosphate Buffered Saline (PBS) (pH 7.2) was obtained from Thermo Fischer Scientific, Waltham, MA, United States of America. Agar plates containing Chloramphenicol ("C-plates") were prepared by Department of Medical Microbiology, TAD, NHLS. A 0.5 McFarland turbidity standard corresponding to 1.5×10^8 cells/mL was used to prepare a C. *albicans* suspension to be used as a stock solution.

2.2. Analytical method

An in-house validated GC-MS isotope dilution blood alcohol analytical method was employed. This involved an *in-situ* esterification of ethanol to form ethyl pentafluorobenzoate, followed by liquid-liquid extraction, and selected ion detection and quantitation against a stable isotope internal standard.

Briefly the procedure was as follows:

The specimen/standard solution (500 µL) was spiked with aqueous internal standard ethanol-D6 (50 µL, 1.164 g/L) and deproteinated with acetonitrile (700 µL). Saturated sodium bicarbonate solution (1000 µL) was added to the supernatant in a clean reaction tube. Pentafluorobenzoyl chloride (5% v/v, 1000 µL) in dichloromethane solvent was added and the mixture was shaken. The organic phase was transferred to a new reaction vessel and after being washed with saturated sodium bicarbonate solution (1000 µL), was dried completely under compressed air, reconstituted with dichloromethane and transferred into a conical insert in a 2-mL GC vial.

The validation figures of merit were as follows.

2.2.1. Linearity

Five non-weighted linear calibration graphs not forced through zero of relative response versus concentration including the above seven concentrations and reagent blank obtained on five separate days yielded an average correlation coefficient of $r^2 = 0.9945 \pm 0.002351$. The 95% confidence intervals for the gradient and intercept were 0.70607 ± 0.00935 and -0.00107 ± 0.03444 respectively. The residuals were examined and it was found that the data was homoscedastic over the calibration range.

2.2.2. Limits of detection and quantitation

A signal for ethanol could no longer be detected at the lowest spiked concentration of 0.000078125 g/L; however, a S/N ratio of 3:1 was obtained at 0.0125 g/L ethanol, and a S/N ratio of 10:1 at 0.025 g/L.

2.2.3. Bias

A non-weighted linear regression bias correction plot was obtained by plotting the average experimental concentrations of the three internal quality control levels (Y) of 0.215 g/L, 0.511 g/L, and 2.951 g/L versus the theoretical concentrations (X) of 0.2 g/L, 0.5 g/L and 3.0 g/L, and had the form $Y = BX + A$. The purpose of this correction plot is to correct for the bias at all concentrations in the linear range of the method, and not only at the discrete concentration levels. The actual bias at each of the three discrete concentrations above was 7.5%, 2.2% and −1.65% respectively. The 95% confidence intervals of the intercept and gradient were determined to be $A = 0.021 \pm 0.024$ and $B = 0.976 \pm 0.014$ respectively. It was thus determined that the method exhibited no additive bias, while it did display multiplicative bias - that is, a bias that is dependent on concentration.

2.2.4. Precision

Precision studies were performed by repeated measurements at the three internal quality control concentration levels of 0.20, 0.50 and 3.00 g/L ethanol in blood. The within batch precisions were found to be 6.5%, 3.8% and 5.5%, and the between batch precisions found to be 11.7%, 9.6% and 9.2% for the three internal quality control levels respectively (n = 20).

2.2.5. Selectivity

The principle of "identification" before "quantification" was applied and ion abundance ratios were used for identification of the ethyl