Stability of Ethanol in Stored Blood: A Validation Study for Blood Alcohol Determination in Victims of Road Traffic Accidents in the Ivory Coast

ABSTRACT

Background: In the Ivory Coast, only the National Laboratory of Toxicology is qualified to complete blood-alcohol testing in road traffic victims or drunk-driving victims. However, there is no legal or existing standardized operational procedure for blood storage and transportation from the emergency units to the National Laboratory of Toxicology.

Objectives: The objectives of this study were to monitor the efficacy of the cold chain from the victim’s bed to the laboratory of toxicology and to assess the stability of whole blood samples with known concentration of ethanol during transportation, storage and pre-analytical phase.

Design and Methods: The cold chain was composed of a cool box, a refrigerator (from +2°C to +8°C) and a freezer (−18°C). Temperature was monitored for each cold device using a digital thermometer. Deviations from targeted or expected temperatures were assessed using control cards. Ethanol concentrations were determined by CPG-FID at baseline, after 24, 48, 72 h and day 15 for blood controls A) stored at +5°C, B) left at room temperature, C) after 3 freeze and thaw cycles and D) after extraction. Differences between ethanol levels at baseline and at various times were tested by the test of Student and ANOVA.

Results: At each stage of the storage and transportation process, temperature in cool devices remained in the targeted range. We found a statistically significant decrease of ethanol in refrigerated blood sample after 15 days of storage. Ethanol loss was significant at ambient temperature after 5 days and after 3 freeze-thaw cycles. In the supernatant, ethanol was stable up to 72 h.

Conclusion: Regarding our working conditions and storing procedures, alcohol concentration can be accurately measured in ER patients’ blood in a frame time of 5 days, provided the samples are refrigerated.

KEYWORDS ethanol, whole blood, storage conditions, stability

INTRODUCTION

Blood-alcohol analysis is typically performed in driving under the influence (DUI), driving while intoxicated (DWI) investigations and in Road Traffic Accidents (RTA) where people have been critically injured or killed. Most developed countries have “per se” laws that provide a full description of the blood sampling procedure, conditions of storage and analytical conditions based on validated research data and on methods adapted to the field reality.

In the Ivory Coast, the law n°63-527 of December 26, 1963, Article 17, provides that: “Evidence of the state of intoxication or alcoholic dependence will be inferred by the judge from factual findings observed by the police officer and on the basis of blood tests established by a decree in order to determine if the offender submitted to the test is under influence of alcohol or not. In case of presumption of drunk-driving, offence of failing to stop at the scene of the accident, homicide or unintentional injuries,
or a serious traffic offense, the offender will undergo a mandatory blood-alcohol testing.

Moreover, blood alcohol dosage (BAC) determination could be very useful in the determination of the responsibility of the driver or the pedestrian in the road accident by police officers. However, there is a legal vacuum concerning standardized methods and analytical process requirements for the determination of blood-alcohol concentration for forensic purpose. Moreover, the current laws do not provide any instruction for blood sample collection, handling and storage prior to blood-alcohol analysis. Yet, the determination of blood-alcohol concentration and its interpretation in drivers or casualties injured or killed after a Road Traffic Accident (RTA) is not straightforward. The quality and reliability of analytical alcohol results strongly depend on the quality of both preanalytical and analytical steps.

From a forensic perspective, each stage in the blood-alcohol determination, from the taking of blood samples, to the storage and transport of samples, to the final analysis, must be taken into account in authenticating the reliability of the final blood alcohol concentration (BAC) which is reported. This aspect is of fundamental importance, especially in developing countries in which the collection and storage of blood samples are frequently deficient. For instance, delays in shipping frozen samples, power cut or voltage fluctuations of the freezers, frequent opening and closing of the freezers, may result in loss or generation of ethanol in blood samples.

Optimal handling and storage conditions of biological specimens for a delayed blood-alcohol analysis remain to be determined in the context of a low-income country and according to our resources, experience and needs.

In the Ivory Coast, people injured in RTA are sent to the Emergency Room (ER) or the Forensic Institute (IMLA), where a blood sample can be taken for alcohol analysis and forensic diagnosis purpose. Once the blood is collected from subjects involved in a fatal or non-fatal accident, many stages should be passed before blood alcohol analysis is completed.

The present study was conducted in concurrence with the ASMA-CI study designed to assess BAC in drivers and pedestrians injured in an RTA and admitted to the ER of a big university hospital in Abidjan (Ivory Coast, West Africa). In the absence of a legal or an existing procedure for blood storage in ER and transportation from ER to Toxicology lab, we implemented a conservation procedure including the use of a portable cold box, a refrigerator and a freezer.

The primary objective of our study was to evaluate the efficacy of the cold chain from the victims’ bed to the lab of Toxicology. A second objective was to assess the changes of ethanol concentration in whole blood depending on most likely scenarios encountered in

**EXPERIMENTAL DESIGN**

**Control of the cold chain**

- Blood samples conservation procedure

  We conducted a study on 893 victims of RTA admitted to ER and forensic institute (ASMA-CI study). During this study, blood samples were collected from victims in the ER and passed through different steps of sample handling, storage and transport before they were analyzed for BAC determination in the laboratory of Toxicology. The routing of blood samples from the patient’s bed to the lab in charge of BAC quantification is fully detailed in the Figure 1. To assess the stability of ethanol in whole blood samples collected during the study, we introduced blood controls with a known concentration of ethanol in the chain of conservation along with blood samples collected from patients. This was done to ensure that the controls underwent the same storage and transport conditions as patients’ blood samples.

- Cold Chain Monitoring

  - Cool box in Emergency Room

  There was no conventional cold system (freezer, refrigerator) in ER for immediate storage of blood samples collected from victims. Therefore, we introduce a portable cool box (Cold device 1) equipped with ice packs in the cold chain. Ice packs were frozen at $-20^\circ C$ for 48 h, kept at room temperature for 15 min and placed in the cool box avoiding any contact with blood samples. The target temperature in the cool box was expected to be in the range between $+2^\circ C$ and $+8^\circ C$. The inside temperature in the box was monitored with a digital thermometer Brannan™ (internal sensor: $-20^\circ C$ to $+70^\circ C$; Resolution: 0.1°C; Precision: $\pm 1^\circ C$). Data were manually collected each hour during 2 weeks including three weekends.

  - Refrigerator in the Central Lab of the University Hospital (Cold device 2)

  Every 8 h, samples were transferred from the cool box to a refrigerator, which was located in the Central Lab of the CHU of Yopougon. Temperature in the refrigerator (Medline-LiebherrLKv 3910) was monitored using the same thermometer as previously described. Data were collected each hour for 2 weeks. The target temperature was expected to be between $+2^\circ C$ and $+8^\circ C$.

  - Freezer in the Lab of Toxicology (LNSP)

  The cold device 4 was a Liebherr GP 2733 (232 Liters) freezer equipped with sensors and an integrated system to display temperatures in real time (Cold device 4).
The stability of ethanol in blood samples from road traffic victims

Data were collected each hour for 2 weeks. Target temperature $T \ (°C) = -18°C \pm 2°C$.

- Simulations for blood conservation (ethanol stability in blood study)

Our aim was to assess the effect of sample storage conditions on the level of BAC. To this end, several ethanol standards in whole blood at 1.1 g/l from the same lot (Medichem, Medidrug Ethanol VB-plus, ME 61113) were prepared and submitted to the following experimental conditions:

**Sample A**: 3 ml of ethanol standard was placed into a vacutainer tube containing $K_2$EDTA (12 mg) as anticoagulant and stored in a cool box along with RTA victims’ blood samples for 8 h, before their transfer in the toxicology lab for immediate ethanol quantification by GC-FID (0 h baseline value). Then, the tube was kept under refrigeration between +2 and +8°C, and ethanol quantification was realized at 24, 48, 72, 120 h, and finally at day 15.

**Sample B**: 3 ml ethanol standard added with $K_2$EDTA was transferred to the toxicology lab after 8 h storage in a cool box in ER as previously described. The 0 h baseline BAC was determined, and the tube was left at room temperature on the laboratory bench at 18°C ± 1°C. BAC was measured by GC-FID at 24, 48, 72, 120 h and 15 days after storage at room temperature.
Sample C: 3 ml ethanol standard, which has been treated the same way as RTA victims’ samples, was received by the lab of Toxicology. At this stage, the control underwent a baseline BAC determination, followed by three freezing (24 h at −18°C) and thaw (room temperature) cycles. BAC was determined after each freezing/thaw cycle.

Sample D: We realized 10 extractions from the blood ethanol standard and alcoholic extracts were stored in 10 vials. Extracts were injected on our GC-FID system after 0 (baseline), 24, 48, 72, 120 h and 15 days conservation (2–8°C).

**BAC determination method**

- **Materials and reagents**

  A SHIMADZU 14A Gas Chromatography System equipped with a Flam Ionization Detector was used for GC/FID quantification. The GC was coupled with a 180 × 0.2 cm capillary column packed with 100–120 mesh Porapak Q.

  Human whole blood standards 1.1 g/l and 0.5 g/l were purchased from Medichem (Medidrug Ethanol VB-plus, ME 61113 and ME 61053, 10 × 3 ml, lot AS021023-1). Normal propanol was used as Internal Standard (IS). Trichloroacetic acid (TCA) was used for the precipitation of blood proteins. To prepare the extraction solution, 300 ml of TCA (100 g/l) was added to 30 ml of n-propanol (4 g/l). Ethanol calibrating solutions (0.5, 0.8, 1.0, 1.5, 2.0, 3.0 and 4.0 g/l) were prepared using a commercially ethanol solution (ethyl alcohol 96.2°, RPE) purchased from Carlo Erba and spiked with the IS. A negative control solution was prepared using MilliQ water (Millipore™) and IS.

- **Sample preparation and extraction**

  The procedure for whole blood extraction was adapted from the method developed by Szymanowicz et al. Briefly, 250 µl of whole blood was added to 500 µl of TCA + IS mix. The sample was shaken for 10 s, followed by centrifugation at 3500 rpm and +4°C for 10 min. Le supernatant was manually injected on the GC column in triplicate.

**Analytical conditions**

GC-FID analytical parameters were as follows: column temperature: from 140°C to 220°C, 10°C/min; injection port temperature: 200°C; detector temperature: 250°C; carrier gas (helium, 99.9999%, Air Liquide) flow: 2.5 ml/min; injection volume: 1µl.

**Validation of the analytical method**

The analysis of BAC by GC-FID method was validated by assessing the precision, accuracy, recovery, linearity and reportable range, limit of detection (LOD) and limit of quantification (LOQ). This general validation scheme was expanded to include the stability of ethanol in the biological matrix.

**Statistical analysis**

The results on temperature monitoring are presented as mean ± standard deviation (SD) and relative standard deviation (RSD). Concerning validation parameters for GC-FID method: fidelity and accuracy were expressed as RSD; recovery was reported as % of measured BAC and theoretical BAC; linearity as determination coefficient R²; the determination of LOD and LOQ was based on the calibration curve approach using the residual SD.

Differences between the baseline BAC and series at various times were tested by the Student t test and ANOVA. The difference was statistically significant if P < 0.05.

**RESULTS**

**Monitoring of blood cold chain**

During the experimentation, the temperature inside the cool box (Cold device 1) remained all the time in the targeted range of +2 to +8°C as shown in the Figure 2A. The mean temperature over 15 days was 3.6°C ± 0.56°C. Concerning the storage of control blood samples in the Central Lab refrigerator (cold device 2), there was no breach in the cold chain throughout the study period (Fig. 2B). The mean temperature recorded was 4.5°C ± 0.76°C. In the Lab of Toxicology, where blood samples were stored in a freezer for postponed BAC analysis, temperatures remained very stable around −18°C ± 1°C during all the study period.
Analytical method validation

Our results indicate that the precision of the quantification method was high as confirmed by a RSD = 4%. The accuracy was also very good with an RSD = 2.5%. The recovery of ethanol in whole blood was found to reach 93–95% and 93–94% for theoretical concentrations of 1.1 g/l and 0.5 g/l, respectively. The linearity of the calibration curve was very good with R² greater than 0.999, and the curve was tested to be linear between 0.5 g/l and 4.0 g/l. The LOD was 0.045 g/l and the LOQ was 0.146 g/l.

In vitro stability of ethanol in whole blood

This stability study was carried out to assess the effect of different storage conditions and sample manipulation on the BAC. The results are compiled in Table 1. The mean BAC in whole blood for samples A stored between +2°C and +8°C decreased significantly (P < 0.05) from the baseline value after 15 days of storage (−0.07 g/Lie. −6.9%). At ambient temperature (18°C ± 1°C), the decrease of ethanol in blood (sample B) was statistically significant (P < 0.05) after 5 days of storage (−0.05 g/Lie. −4.9%). A statistically significant decrease of ethanol in whole blood (sample C) was observed after 3 freezing/thaw cycles (ethanol loss = 0.03 g/Lie. −2.97%). Ethanol present in the supernatant obtained after the sample preparation and extraction (sample D) was stable up to 72 h of storage between +2°C and +8°C. Beyond this limit, we observed a significant decrease of 0.05 g/l (−5%) and 0.07 g/l (−7%) after 120 h and 15 days of storage, respectively.

DISCUSSION

This study was implemented to address the lack of standardized operational procedure concerning the storage and analysis of BAC in road users involved in an RTA in the Ivory Coast. Due to our specific climatic conditions and limited financial and human resources, it appeared very important to validate a procedure for blood storage and analysis that takes into account our specificities and is in compliance with forensic standards.

In a first step of our study, we fully monitored the blood cold chain implemented to secure a good conservation of blood samples collected on RTA victims for delayed blood alcohol determination in our Toxicology Laboratory. The mean duration for blood samples storage and transportation, from the patient’s bed to the lab of toxicology, was approx. 80 h. Due to our limited logistic facilities, blood samples collected in the ER could not be transferred immediately or one at a time to the unique Lab of Toxicology which is located 35–40 km away from ER.

We demonstrated that at each stage of the storage (in ER and hospital laboratory) and transportation process, no breach was observed in the cold chain during the study period. Also, no significant change in alcohol concentration was observed during this period. The present procedure was experimented in a controlled study context, but could be easily adapted to our daily practice. The main challenge is the traceability of samples which requires a good knowledge of and compliance with storing procedures. The availability of clear written procedures and the implementation of a quality control approach in our lab allowed us to achieve this goal.

The next important question was then to evaluate the stability of blood ethanol over time and for various temperatures of storage in the National Toxicology Lab. The stability of blood ethanol is a very important issue as samples are required to be stored for more than 3 days before the analysis is completed. Moreover, power failures are frequent during certain periods of the year in our lab, highlighting the need to address the stability of blood ethanol at ambient temperature and after thawing process. Consequently, we tested the stability of ethanol using sterile human blood standards with a known BAC (1.1 g/l), which were handled.

Table 1 Variations of mean BAC (g/l) in blood ethanol standards (1.1 g/l) for various storage temperature and time (stability study).

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Sample A Reference (2 – 8°C)</th>
<th>Sample B Ambient temperature (18 – 20°C)</th>
<th>Sample C Freeze/thaw (−18°C)</th>
<th>Sample D Sample extract 2 – 8°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.004 ± 0.034</td>
<td>1.009 ± 0.035</td>
<td>1.009 ± 0.035</td>
<td>1.000 ± 0.049</td>
</tr>
<tr>
<td>24</td>
<td>0.993 ± 0.036</td>
<td>0.989 ± 0.028</td>
<td>0.991 ± 0.040</td>
<td>0.988 ± 0.044</td>
</tr>
<tr>
<td>48</td>
<td>0.990 ± 0.041</td>
<td>0.978 ± 0.037</td>
<td>0.987 ± 0.045</td>
<td>0.994 ± 0.040</td>
</tr>
<tr>
<td>72</td>
<td>0.990 ± 0.055</td>
<td>0.976 ± 0.038</td>
<td>0.974 ± 0.034**</td>
<td>0.979 ± 0.031</td>
</tr>
<tr>
<td>120</td>
<td>0.990 ± 0.046</td>
<td>0.955 ± 0.024**</td>
<td>–</td>
<td>0.944 ± 0.028**</td>
</tr>
<tr>
<td>15 days</td>
<td>0.931 ± 0.028**</td>
<td>0.899 ± 0.029**</td>
<td>–</td>
<td>0.927 ± 0.029**</td>
</tr>
</tbody>
</table>

P-value* <0.05 <0.05 <0.05 <0.05

*Comparison of mean BAC for each time period with the initial T₀ using student t-test and ANOVA.
stored and analyzed exactly in the same conditions as RTA victims’ blood samples (Fig. 1). We purposely avoided the use of patients’ samples that may result in generation or loss of ethanol due to diseases or comorbidities like infections, septicemia, diabetes or mycosis/ candidiasis. Thus, our results are not affected by inter individual variability. The current experiment was conducted in an attempt to improve on previous studies by analyzing known ethanol concentration sample which underwent exactly the same conditions as real blood samples collected in ER for forensic analysis purposes. The BAC measured on the day of sample reception in our lab was considered as baseline for comparison with other BAC measured after re-analysis under different storing conditions.

We did not observe any generation of alcohol during the study period. However, we demonstrated a statistical significant decrease of BAC after 15 days when the sample was stored between +2°C and +8°C. This decrease (6.9%) was also analytically significant as the precision of our analytical method by GC-FID gave a RSD = 4%. We observed a significant decrease in ethanol concentration after 5 days of storage at room temperature. Our findings are not consistent with those reported by Winek and Paul (1983) who concluded that BAC analysis could be delayed for as long as 14 days without significant variations in ethanol, regardless of whether the samples were refrigerated or not. Our experiment also revealed that at ambient temperature, the loss of ethanol after 15 days was 11% compared to the day 0. Mandic-Radic (2007) found a significant decrease of 22.4% after 14 days at ambient temperature in a blood ethanol stability study conducted in Belgrade (Serbia). In this later study, the exact room temperature was not specified and the BAC determination method was different, which could explain the discrepancy between the two studies. In our study, although the toxicology lab was located in sub-Saharan Africa where temperatures usually vary between 25°C and 35°C depending of the season, the room temperature was set to 21°C by air conditioner, and the average ambient humidity was 85%.

Effect of thawing on blood-alcohol concentration is rarely discussed in the literature. We hypothesized that condensation resulting from consecutive freezing and thawing cycles may induce significant dilution of ethanol in blood samples. The BAC decrease (2.97%) was statistically significant after 3 freeze and thaw cycles, but not analytically significant.

Our lab has one single GC-FID, which is neither equipped with a headspace nor with an auto sampler. For this reason, all blood samples have to undergo a sample extraction and preparation phase prior the injection on GC-FID. Furthermore, in absence of autosampler, individual injections of a batch of samples are time consuming, and sometimes alcohol extracts need to be stored in the freezer until injection. Consequently, the stability of the supernatant was assessed in this study to address this possible source of error in measurement of BAC. The supernatant obtained after blood sample preparation and extraction was stable up to 72 h between +2°C and +8°C. The supernatant was stored in a 1.5 ml brown vial. The decrease in BAC was statistically and analytically significant after 5 days (5%), and the loss was 7% after 15 days.

Previously published studies have concluded that the stability of ethanol in stored blood samples strongly depends on three main factors, such temperature and length of storage, volume of air above blood and tightness of stoppers for the collecting tubes. More specifically, Jones (2007) showed that repeatedly opening of tubes of blood to remove aliquot for analysis resulted in a greater loss of ethanol. The author suggested that ventilation with ambient air plays a role in the degradation of ethanol in blood samples. Saracevic et al. demonstrated that blood samples are acceptable for alcohol determination within 2 h after removing the stopper, when stored at room temperature. Therefore, repeatedly opening and closing of the containers may explain alcohol loss in our study, especially at room temperature. Another plausible mechanism to explain the degradation of ethanol is non-enzymatic oxidation reaction that involves oxyhemoglobin within the erythrocytes. However, this later hypothesis could not explain the significant alcohol decrease observed in the supernatant obtained after blood sample extraction. The differences observed between our results and the literature, teach us that the stability of ethanol in blood samples is strongly dependant on environmental and working conditions in different laboratories. Consequently, each laboratory should establish its own criteria for reliable storage given actual conditions in that laboratory as recommended by Mandic-Radic.

CONCLUSION

Regarding our working conditions and storing procedures, we recommend that blood samples collected for BAC determination in the National Lab of Toxicology should strictly be analysed within 14 days after collection. This holds true only when blood samples are stored in freezer. BAC could not reliably be measured when blood sample has been stored for more than 5 days at room temperature. Following sample preparation and extraction phase, the injection of the supernatant on GC-FID could be delayed no longer than 72 h, provided the supernatant is refrigerated between +2°C and +8°C. These data provide interesting basis for the development of a standard operational procedure for BAC determination that takes into account both our specificities and forensic toxicology standards.

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The stability of ethanol in blood samples from road traffic victims

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AUTHORS CONTRIBUTION

Each author identified in the manuscript has contributed significantly to the work and agrees to the submission. Diakite, Gadegbeku, Laumon, and Malan designed the study. Diakite collected and analyzed the data and did toxicological validation and analysis. Laumon, Gadegbeku, Malan and Dano critically reviewed the paper. Diakite wrote the draft of the paper.

REFERENCES