Blood biochemical markers could improve the reliability of postmortem alcohol analyses

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Abstract: Quantification of alcohol levels in postmortem blood after road-traffic crashes is of importance for the determination of the driver’s state of inebriation at the time of death. However, in certain circumstances such results are difficult to interpret because of the necrokinekinetics phenomenon, which involves changes in drug concentration at different rates over time in different corpse sites, happening at early stage after death. The present study shows cases of alcohol-intoxicated men that died due to head or chest traumatic injuries in road-traffic crashes. Toxicological results showed differences according to sampling site in blood alcohol concentrations in addition to alterations in blood or serum parameters like hematocrit, water content, pH and cytosolic enzyme activity. It was found, as expected, a progressive impairment in cellular permeability and dehydration in central blood cells, although this was accompanied by a diminution in alcohol concentration. However, peripheral blood showed hematocrit, water content and cytosolic enzyme activity values similar to those observed in living persons. Such findings suggest that blood alcohol levels obtained from peripheral sites constitute a reliable indicator of the state of inebriation at the time of death and in the early postmortem stage, as long as blood or serum parameters do not exceed normal limits. We propose that blood samples used for alcohol determination must be obtained from peripheral sites and may be accompanied by a measure of a blood biochemical marker in order to provide reliability to toxicological findings and to minimize detrimental effects of decomposition processes in the early postmortem stage.

Key Words: peripheral blood, early-stage postmortem, dehydration, autolysis, blood alcohol.

One of the causes of death related to acute alcohol poisoning is attributed to traumatic injuries resulting from a road-traffic crash [1]. Recently, in México most of deaths related to road traffic accidents were associated to driving under the influence of alcohol [2]. Measuring and interpreting the concentration of alcohol in blood and other biological specimens are routine procedures in forensic medicine and toxicology [3]. The interpretation of alcohol levels typically focuses more on the discussion of alcohol’s impairment effects on human performance and behavioural toxicity, than on its overt physiological toxicity [4]. Difficulties arise when the alcohol concentration of a postmortem blood specimen is interpreted and conclusions are drawn about a person’s state of inebriation at the time of decease in spite of the manner and cause of death.

A major problem associated with postmortem alcohol analysis is the risk that alcohol, at least in part, can be generated or destroyed between the time of death and the time of the autopsy [3]. It is a common practice

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that alcohol generation can be avoided by using blood collection tubes containing preservative in order to suppress enzymatic and microbial activity [5]. In Mexico, usual forensic practices also do not allow postmortem alcohol generation in the early postmortem stage, because physicians are used to execute autopsy as early as a corpse is found and additionally because toxicology routine analysis are performed immediately after the body fluid has been sampled.

However, it is difficult to establish blood alcohol destruction without analysis of the sample and subsequent comparison with antemortem analysis or simultaneous results from another sampling site. This situation makes it necessary to sample blood from various vessels to confirm a change in drug concentration over time after death; this phenomenon has been termed necrokinetics [6, 7]. The mechanisms of change in drug concentrations after death can be considered comparable to the physiologic mechanisms that drive pharmacokinetics. Changes appear to occur secondary to passive diffusion within the body as cell function ceases, and cellular integrity is lost [8]. Trauma is one condition that can have significant influence in the degree of change affecting drugs following death [9].

Variations in postmortem alcohol concentration have been reported between the left and right chambers of the heart [10], between pulmonary and femoral veins [11], and even between two non-central vessels, like femoral and subclavian [12]. These changes are generally attributed to early alcohol diffusion from the stomach or other reservoirs to surrounded tissues in the torso [13], which explains the higher alcohol concentration in central blood sites than in peripheral ones. Nevertheless, we have found several cases of men died by head or chest trauma where a peripheral site shows a blood alcohol concentration higher than a central site, and consequently, the phenomenon cannot be explained by a diffusion process, and it is mandatory to investigate another causes related to alcohol destruction associated with decomposition processes in the early postmortem stage. The goal of the present study is to demonstrate how a group of corpses from men that died by trauma injuries show differences in blood alcohol concentration parallel to alterations in blood or serum parameters like hematocrit, water content, pH and cytosolic enzyme activity according to sampling site, i.e. central blood versus peripheral blood.

**MATERIALS AND METHODS**

**Case identification**

This study was carried out with medico-legal cases of deaths that occurred from September 2005 to January 2007 in the central region of the Veracruz state, located in southeast México. The forensic pathologist based on circumstances of death, autopsy findings and toxicological analyses determined cause of death. Eight corpses aged between 20 and 35 years were grouped because all of them showed an unusual peripheral blood alcohol concentration higher than the one found in central blood. All subjects had similar causes of death and suffered from similar injuries, i.e. mechanical death in traffic road crashes due to contusing chest or head trauma without rupture of a large blood central vessel. The criterion used to define the postmortem interval was to include cases only in situations when the time of death was reported by at least one witness with a time frame no longer than several minutes. The time elapsing between death and autopsy was equal or less than 12 h.

**Biological specimens**

Whole blood samples were grouped in central (CB) or peripheral blood (PB). Central blood samples were obtained by opening the pericardial sac and aspirating from the pulmonary vein, while peripheral blood was obtained from the femoral vein, which was dissected and sampled directly. In order to minimize hemolysis, serum samples were immediately obtained by centrifugation at 2,500 rpm (Universal 32, Hettich Zentrifugen). Blood samples were deposited in lavender-cap Vacutainer tubes (K2 EDTA, Becton & Dickinson; NJ, USA) while serum samples were deposited in red-cap Vacutainer tubes (uncoated, Becton & Dickinson; NJ, USA).

**pH determination in serum**

pH as a parameter of postmortem acidification was determined in 200 µL serum samples by using an Orion 720A potentiometer equipped with a Thermo microelectrode at 22°C (±0.5).

**Alanine aminotransferase activity determination in serum**

Alanine aminotransferase (ALAT), a cytosol enzyme, was used as a cellular membrane integrity marker. It was determined in serum samples according to the Reitman & Frankel method [14]. Alanine aminotransferase was measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenyl-hydrazone which was used according to the manufacturer’s instructions (Randox Laboratories Ltd.; Co. Antrim, UK). A serum supplemented blank and a calibration curve was prepared simultaneously with daily determinations consisting of six different concentrations between 9 and 87 U/L of ALAT enzymatic activity. Pyruvate hydrazone absorption was measured by using an UV-Vis Lambda 25 Spectrophotometer (Perkin Elmer Inc., MA, USA) at 546 nm.

**Hematocrit**

Hematocrit as a cellular volume marker was determined using 75 µL capillary tubes (Marienfeld, Germany), which were partially filled with whole blood and centrifuged at 12,000 rpm (Sigma 2-16P). Results were expressed as the percentage volume of the tube that was composed of erythrocytes.
Blood water content

Whole blood water content was used as a marker of dehydration in the early postmortem stage. It was determined by weighing and drying 100 µL aliquots of whole blood samples in an oven at a fixed temperature (60°C) for 12 h. Water content was calculated using the following equation:

\[
\text{Water content (\%)} = \left( \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \right) \times 100 \quad [15]
\]

Blood alcohol determination

1 mL aliquots of whole blood samples were deposited in 22 mL headspace sample vials and supplemented with 78.6 µg of n-butyl alcohol as internal standard (IS). Then they were sealed with PTFE/silicon septum. Ethanol analyses were performed using a headspace gas chromatograph equipped with a flame ionization detector (FID) and a headspace sampler (Autosystem XL; Perkin Elmer Inc., MA, USA). A PE-FFAP capillary column (30 m x 0.53 mm i.d., 1.0 µm film thickness, Perkin Elmer Inc., MA, USA) was used for the GC separation of the analytes. Carrier gas was nitrogen at 1.0 mL/min. The operation conditions were as follows: The injector temperature was 100°C and the detector temperature 150°C. During analysis, the oven temperature program was: 60°C for 5 min. Samples were heated in the headspace glass vials for 8 min at 60°C for equilibration before injection. Chromatograms were recorded and calculations were done with the use of the Turbochrom computer program (Perkin Elmer Inc., MA, USA). Eight-point calibration curves were prepared, with analyte concentration in standard solutions ranging from 158 to 4740 µg/mL for ethanol. Pooled whole blood from donors was used to prepare standard solutions in order to minimize matrix effect.

Statistical analyses

Mean, range and standard error of the mean (S.E.M.) were used as descriptive statistics. In order to evaluate postmortem interval and blood sampling site effects on decomposition parameters we use a two-way analysis of variances.

Concerning blood alcohol concentration, we evaluated only the sampling site effect by using a student t-test for dependent samples. These analyses were carried out using GraphPad Prism (version 6.00; California, USA).

A p value <0.05 was considered statistically significant.

RESULTS

Autolysis markers

Figure 1 A shows findings of a progressive acidification in serum related with postmortem interval (PI). pH in serum samples at PI ≤ 6 h (6.85 ± 0.02 and 7.01 ± 0.15 for CB and PB respectively) was significant higher (F1,13 = 7.0; p < 0.05) than serum samples at 7 h > PI < 12 h (6.38 ± 0.06 and 6.9 ± 0.13 for CB and PB respectively). Also, pH mean values of PB differed significant concerning to CB mean values (F 1,13 = 9.6; p < 0.05).

Moreover, increased ALAT activity in serum was observed according postmortem interval elapsed (Fig. 1B). For PI ≤ 6 h enzymatic activity were 29.95 (range 4.7-68.9) and 8.1 (range 1.3-21.5) U/L for CB and PB respectively while results with respect to postmortem interval between 7 and 12 h were 105.9 (range 92.3-126.7) for CB and 13.8 (range 4.5-25.0) U/L for PB. There were significant effects of sample site collection (F 1,9 = 12.2; p < 0.05) and postmortem interval (F1,9 = 6.3; p <0.05) on
the enzymatic activity of this cellular integrity marker.

**Dehydration markers**

Figure 2A shows that hematocrit raised with time elapsing between death and autopsy. Cases within 6 h of postmortem interval were 54.9 ± 2.4 and 49.9 ± 3 for CB and PB respectively. Next lapse showed 58.7 ± 5 for CB and 49.5 ± 0.8 for PB. It was only observed a significant difference between blood sampling sites (F₁,₁₃ = 4.8; p < 0.05).

By contrast, blood dehydration did not differ in cases where PI was between 7 and 12 h (74.1 ± 0.8 and 78.3 ± 0.8 for CB and PB respectively) with respect to PI ≤ 6 h (75.2 ± 0.3 for CB and 78.1 ± 0.7 for PB). By contrast, blood water content was significant lower in CB (Fig. 2B) concerning to PB (F₁,₁₃ = 27.1; p < 0.05).

**Blood Alcohol Concentration**

The standard curve exhibited a rectilinear relationship between concentration and detector response over a range of 158 to 4,740 µg/mL with a coefficient of correlation exceeding 0.99. Blood alcohol concentration (BAC) was obtained by calculating chromatographic peak areas and by using internal standard method. Blood alcohol concentration according postmortem interval and sampling site is shown in Table 1.

Because the heterogeneity observed in BAC among the distinct cases, there was no possible to evaluate statistically the postmortem interval effect. In spite of this, we conducted a student t-test for dependent samples in order to test for site dependent differences. It was found a significant lower BAC in CB (1800 ± 249 µg/mL) concerning to PB (2140 ± 296 µg/mL) from all the corpses sampled (t (7) = 3.25; p < 0.05).

**DISCUSSION**

Recently, numerous authors have been focused in supravitality studies of organs and tissues in the early stage after death in order to explain postmortem redistribution of biomarkers, the cause and process of death and to give an accurate postmortem interval index [16, 17]. They argue that supravitality sustains cellular homeostasis in the first hours after death because there could be enough adenosine triphosphate via glycogenolysis [18]. These findings showed the importance of analyzing decomposition processes at various corpse sites and their influence on analyte stability in the early postmortem stage. Bardale et al. [19] showed that for an approximately 6-hour postmortem interval, no morphological changes were observed in the epidermis and the dermis of human anterior chest. Likewise in blood, Dokgöz et al. [20] indicated that neutrophils, eosinophils and monocytes did not lose their normal morphology during the first 6 h after death. In our study, differences between postmortem lapses in relation to pH and ALAT enzymatic activity findings suggest that cellular permeability impairment begins to appear since 6 h after death and it is exacerbated in central blood. We confirm that the six-hour intervals...
were established conveniently because the significance of the postmortem lapse effect on the serum parameters assayed.

Moriya & Hashimoto [21] stated that body fluids pH decline in the early postmortem stage due to lactate accumulation as a result of anaerobic metabolism. Moreover, Garg et al. [22] indicated a ten-fold increase in serum ALAT activity after 6 h after death. We hypothesize that surrounding tissues or the pulmonary vein itself release their intracellular components into the blood earlier than blood in peripheral vessels, resulting in exacerbated acidification and autolysis in central blood. Such findings confirmed that early-stage decomposition processes cause differential changes according to site and time elapsing between death and blood collection as mentioned by other authors.

It is remarkable that, according to the normal range for a live subject, peripheral blood did not exceed the upper limit in serum ALAT activity [14], which demonstrates tissue integrity in the first twelve hours after death.

Concerning blood dehydration markers, we found that while hematocrit mean values in central blood were higher than normal range values for live male adults i.e. 42-50% [23], peripheral blood values did not exceed the upper limit.

Similarly to hematocrit, water content mean values in peripheral blood were close to the normal mean value of fresh blood, i.e. approximately 80% [3].

Considering the decomposition processes that corpses undergo during the early hours after death, we suggest that peripheral blood is more resistant to detrimental effects as visualized by the significant differences in hematocrit, water content, pH and cytosolic enzymatic activity with respect to central blood. An analogy to such differences between central and peripheral sites consist in measuring body cooling rate, which is lower in the rectum (central site) with respect to ocular globes (peripheral site) as a result of the storing of heat in the central tissues and their residual heat-generating metabolism, which continues for some time after death [24].

There were no other peaks in the chromatograms besides exogenous ethanol and the internal standard used, which confirmed the absence of other volatile substances such as putrefaction products in each case (data not shown). Therefore, we consider blood alcohol as only exogenous and any change in blood alcohol concentration could be attributed in turn to phenomena occurring in each corpse during the postmortem interval. According to the differences in alcohol concentration between blood sampling sites, we propose a diminution in alcohol concentration in central blood as a result of dehydration and/or by enzymatic breakdown. In the first proposition, alcohol loss from a blood vessel is parallel to water loss, which is indicated by an increase in hematocrit and a decrease in blood water content.

In the second hypothesis, alcohol breakdown by catalytic activity is facilitated by a widespread leakage of cellular enzymes and macromolecules into the extracellular space as a result of increased membrane permeability [25] and an intensified autolysis subsequent to traumatic lesions, which releases cytosolic enzymes like alanine aminotransferase and alcohol dehydrogenase inclusive. This leakage is also confirmed by plasma acidification, which is exacerbated in central blood.

Skopp et al. [26] argued that postmortem permeation process through a vascular wall depended on autolysis, initial drug concentration, molecular structure, and on the orientation of solute flux, which in this case goes to outside the vessel. Both process, e.g. autolysis and dehydration, can explain such phenomenon of a higher blood alcohol concentration in peripheral site with respect to central site. But, in order to elucidate which process is preponderating, it is mandatory to evaluate autolysis in blood as well as in tissues, and also is necessary to use an animal model of postmortem dehydration in subsequent experiments.

CONCLUSION

Considering the postmortem stability of femoral blood with respect to blood of pulmonary vein in death cases occurred by head or chest severe trauma, we suggest that blood samples used for alcohol determination must be obtained from peripheral sites and may be accompanied by a measure of a blood homeostasis marker, such as hematocrit, blood water content or cytosolic enzymatic activity; in order to provide reliability to toxicological findings and to minimize detrimental effects of autolysis and dehydration.

Evaluation of such detrimental effects on alcohol concentration needs to be investigated further in future experiments using animal models and by controlling simultaneously certain variables like alcohol dose and postmortem interval.

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Ethical approval. This study has been carried out in accordance with The Code of Ethics of the World Medical Association. In Mexico, Ministerio Publico Agency is mandated by statute to investigate prescribed categories of reportable deaths; including those that are sudden, unexpected, violent or unnatural. Federal rule
establishes an official order from this legally authorized representative as a requirement of informed consent to investigate human corpses without need of further permissions [27].

Conflict of interest. There are no conflicts of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence this study.

References

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