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Journal: Forensic science international

Year: 2018 Feb

Issue: 283

Pages: 21-28

DOI: 10.1016/j.forsciint.2017.12.009

Elsevier Editorial System(tm) for Forensic Science International

Manuscript Draft

Manuscript Number: FSI-D-17-00730R1

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Article Type: Original Research Article

Keywords: methemoglobin, postmortem, hemoglobin

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Abstract: Hemoglobin is the protein in red blood cells that carries and distributes oxygen to the body. Methemoglobinemia is a blood disorder in which an abnormal amount of methemoglobin (MetHb), a form of hemoglobin (Hb), is produced from either inadequate MetHb reductase activity or too much MetHb production or by exposure to oxidizing agents. This could lead to anoxia and death if it is not treated. However, this parameter has not been investigated as a valid post-mortem indicator because random MetHb levels have been observed in various studies: MetHb increases can be observed due to autoxidation during storage, and MetHb decreases can be observed due to MetHb reductase or microbial activity in post-mortem samples. MetHb variations can also come from the blood state and can interfere in the optical measurements of MetHb. We have studied the post-mortem MetHb concentrations according to various storage conditions. Based on our results, both the post-mortem delay and the delay before analysis should be reduced whenever possible to avoid changes in MetHb. If the analysis is delayed for a short period of time (two weeks), the blood sample taken at autopsy should not be frozen but collected in EDTA preservative and stored under refrigeration (4-6°C) until analysis. If the analysis is delayed for a longer period (more than two weeks), the blood sample should be frozen with cryoprotectant at -80°C or -196°C.

Stability of postmortem methemoglobin: artifactual changes caused by storage conditions

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Hemoglobin is the protein in red blood cells that carries and distributes oxygen to the body. Methemoglobinemia is a blood disorder in which an abnormal amount of methemoglobin (MetHb), a form of hemoglobin (Hb), is produced from either inadequate MetHb reductase activity or too much MetHb production or by exposure to oxidizing agents. This could lead to anoxia and death if it is not treated. However, this parameter has not been investigated as a valid post-mortem indicator because random MetHb levels have been observed in various studies: MetHb increases can be observed due to autoxidation during storage, and MetHb decreases can be observed due to MetHb reductase or microbial activity in post-mortem samples. MetHb variations can also come from the blood state and can interfere in the optical measurements of MetHb. We have studied the post-mortem MetHb concentrations according to various storage conditions. Based on our results, both the post-mortem delay and the delay before analysis should be reduced whenever possible to avoid changes in MetHb. If the analysis is delayed for a short period of time (two weeks), the blood sample taken at autopsy should not be frozen but collected in EDTA preservative and stored under refrigeration (4-6°C) until analysis. If the analysis is delayed for a longer period (more than two weeks), the blood sample should be frozen with cryoprotectant at -80°C or -196°C.

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Introduction

Methemoglobin (MetHb) is a form of the oxygen-carrying metalloprotein hemoglobin (Hb), in which the iron in the heme group is in the Fe^{3+} (ferric) state, not the Fe^{2+} (ferrous) state of normal Hb. MetHb cannot bind oxygen and is a factor in asphyxia, which can lead to lethal anoxia. Methemoglobinemia is characterized by varying degrees of cyanosis due to increased concentrations of Hb, which contains oxidized iron. This phenomenon can therefore be easily diagnosed and analyzed in living individuals. [1-5]

Small concentrations of MetHb can be found in the blood of normal individuals. Increased concentrations of MetHb can be consecutive to the action of certain chemicals, such as nitrates [6], nitrites [7], alkyl nitrites such as poppers [8-12] and phosphide [13,14]. Certain drugs are also known to cause methemoglobinemia [15], including benzocaine, prilocaine or lidocaine, which are topical anesthetics that are used as cocaine adulterants and during bronchoscopy, laryngoscopy, or upper gastrointestinal endoscopy [16-20]. Finally, methemoglobinemia can occur in individuals with specific genetic defects, such as a lack of NADH diaphorase [21], NADH MetHb reductase (autosomal recessive deficiencies in cytochrome b5 or cytochrome b5 reductase) [22] or an aberrant form of Hb [23]. As a result, methemoglobinemia results from inadequate enzyme activity, too much MetHb production or exposure to oxidizing agents.

Although the postmortem measurement of MetHb is not considered a valid indicator of antemortem methemoglobinemia [24], other studies have identified elevated postmortem MetHb as a fatal hypoxic cofactor [25]. Exposure to oxidizing vehicles' exhaust fumes (nitrous oxides) [26], ingesting nitrites and nitrates [27-29], or poisoning with alkyl nitrites ("poppers") by ingestion or inhalation [30], can lead to fatal MetHb increases. However, only cases describing a MetHb measurement at bedside or rapidly after blood sampling on living persons are applicable because the storage conditions used for postmortem blood appear to be critical to avoiding MetHb variations. Indeed, the temperature, storage time, preservative, and quality of blood (linked to post-mortem delay and degree of body alteration) can all influence the postmortem MetHb [31-33].

The aim of this study is to document the stability of postmortem MetHb and identify the storage parameters that could eventually change its value. Storage guidelines are needed in order to analyse post-mortem MetHb. MetHb has been identified as very useful to diagnose clinical pathologies or health events and scientists have hypothesized the MetHb relevance in post-mortem conclusions. However, the post-mortem relevance of MetHb cannot be studied if it is biased by inadequate storage conditions.

Materials and Methods

Blood specimens and storage conditions

Postmortem specimens of femoral and cardiac blood were collected during autopsies of deceased individuals at the University Center of Legal Medicine, Lausanne Hospital. The selection of subjects was only done according to the postmortem interval (PMI). Four categories of blood measurements were performed and constituted the inclusion criteria: PMI < 12 hours (n = 4), 12 < PMI < 24 hours (n = 4), 24 < PMI < 48 hours (n = 6) and PMI > 48 hours (n = 4). However, depending on the experimental point over the time, MetHb values were not available because of measurement problems/artifacts due to the sample states and analysis dates (weekend). As result, the exclusion criterion was the impossibility to obtain a MetHb measurement. The number of cases in each category was a limiting factor because more cases would be needed to increase the statistical strength of the results. However, the random inclusion of cases received in our center determined the study design because peripheral and cardiac blood samples from cases with PMI < 12 hours are very rare, as are analyzable samples coming from cases with PMI > 48 hours.

The samples were collected in 2.7 mL tubes containing preservatives, such as ethylenediaminetetraacetic acid (EDTA) or sodium fluoride, and without any preservative (native blood). The air dead volumes in the tubes were not greater than 50 %. The MetHb saturation was measured at the reception date (D0) on the day of the autopsy, then after 1 (D1), 2 (D2), 7 (D7), 14 (D14) and 28 days (D28) of storage at ambient

temperature in a dark place (close to 20°C), at refrigerated temperature (+4°C) or at freezer temperature (-20°C). After analysis, all samples were stored again in their respective storage conditions. For refrigerated samples, the MetHb measurements performed at D1, D2, D7, D14 and D28 correspond to measurements done on samples subjected to, respectively, 1, 2, 3, 4 and 5 opening/closing cycles. Similarly, the MetHb measurements performed at D1, D2, D7, D14 and D28 correspond to measurements done on samples subjected to, respectively, 1, 2, 3, 4 and 5 thawing/freezing cycles. This was deliberately done to mimic laboratory conditions because a blood sample may have been thawed/frozen and opened several times for drug or alcohol analyses before being used for MetHb analysis.

CO-oximetry

MetHb saturation was measured by CO-oximetry with an Avoximeter 4000 from ITC (Edison, USA) and the analyses were done according to the requirements of manufacturer defining the operational and valid ranges. No sample preparation is required, and analysis is quickly accomplished by injecting the sample into a disposable cuvette and inserting the cuvette into the instrument. The instrument illuminates the sample with multiple wavelengths, records the optical density at each of the wavelengths, and computes the results. Optical quality controls were performed according to the standard procedures for the instrument before each batch of analyses. The MetHb reportable range was 0 – 85 %, with an accuracy of ± 1.5 % and a precision of 0.7 %, according to the manufacturer. Hemolysis, bilirubin fetal hemoglobin, and indocyanine green dye can cause interference, but it occurs in less than 1 % of all analyses.

MetHb analyses were directly performed on blood stored at ambient temperature and refrigerated. Frozen samples were kept at room temperature until they were completely thawed before analysis. However, refrigerated and thawed samples were analyzed when their temperature was between +4 and +20°C.

Results

A multifactorial analysis of variance was done on the MetHb measurements to identify the most influential parameters during the storage of blood samples. The results compiled in Table 1 show that regardless of the postmortem delay and analysis date (and the number of opening/closing and/or thawing/freezing cycles), the origin of blood (cardiac or peripheral) is not significantly responsible for the changes in MetHb during sample storage. Consequently, MetHb measurements can be done on blood, independent of its anatomical origin. Moreover, the results illustrate that except for the outlying measurements on Day 1, post-mortem delay has a strong influence on MetHb variations and that except for the outlying measurements on Day 2, the nature of preservative is influential. Concerning the temperature, except for the measurements obtained on D0 (which is normal because all of the samples were at the same temperature), this parameter was identified as crucial to explaining the MetHb saturation modifications during storage. Concerning the interactions at orders 2 and 3 between the different factors, those implying the non-influential "blood origin" factor are not as influential as expected, whereas the interactions among post-mortem delay, temperature and preservative cannot be neglected, thus strengthening the role that temperature plays in the MetHb variations during storage.

The results of MetHb according to the blood origins (cardiac blood/peripheral blood), preservatives and temperatures are displayed in Figure 1 (post-mortem delay < 12 hours), Figure 2 (12 < post-mortem delay < 24 hours), Figure 3 (24 < post-mortem delay < 48 hours) and Figure 4 (post-mortem delay > 48 hours), according to the analysis date (and respective number of opening/closing and/or thawing/freezing cycles).

Except for one case in the category of 24 < post-mortem delay < 48 hours and another in the category of post-mortem delay > 48 hours, all of the MetHb measurements on Day 0 are similar and < 5 %. A complete survey of post-mortem MetHb should also be performed once the influential storage parameters for MetHb are identified because the post-mortem delay, cause and circumstances of death could cause initial increases in MetHb. However, due to limitations, this point has not been investigated in this study.

Considering the categories of post-mortem delay < 12 hours (Figure 1) and 12 < post-mortem delay < 24 hours (Figure 2), storing blood with EDTA and sodium fluoride at refrigerated temperature or without preservatives at room temperature were found to be the best ways to prevent increases in MetHb over time.
Considering the category of 24 < post-mortem delay < 48 hours (Figure 3), storing blood without preservatives at refrigerated temperature was found to be the best way to prevent MetHb increases over time. After two weeks and four opening/closing cycles, MetHb was still below 5 %, as at Day 0.
Considering the category of post-mortem delay > 48 hours (Figure 4), only samples with EDTA or without preservatives stored at refrigerated temperatures can be considered to have prevented MetHb increases over time because after four weeks and five opening/closing cycles, MetHb was still below 10 %.

Discussion

Cardiac vs. Peripheral blood

The results clearly indicate that the MetHb measurement is not influenced by the anatomical origin of blood. For the same considered body, regardless of the post-mortem delay and storage parameters used, the MetHb saturation can be considered similar between cardiac and peripheral blood. However, for both types of blood, with an increase in the storage delay before analysis, opening/closing and/or freezing/thawing cycles, the blood state becomes problematic for obtaining optical measurements by using a CO-oximeter because of artifact generation.

Preservative

To limit the potential oxidation of blood by air during the sampling and different opening/closing cycles, the dead volumes in tubes were reduced to a volume between 0 and 50 % of the total tube volume. However, the

addition of preservatives to the blood tube before storage could be important for understanding the MetHb variations. The role of preservatives in MetHb variations should not be interpreted without considering the thermal storage conditions, as shown in Table 1. For short post-mortem delays (< 12 hours), the nature or the presence of a preservative does not seem to influence MetHb saturations, but the samples must be refrigerated. For a post-mortem delay between 12 and 24 hours, storage with EDTA or sodium fluoride requires a refrigerated temperature, whereas the absence of preservatives seems to be tolerated at room temperature. For a post-mortem delay between 24 and 48 hours, storage with EDTA or sodium fluoride does not seem to be recommended because MetHb variations have been observed, whereas the absence of preservatives requires refrigerator storage. Finally, for post-mortem delays > 48 hours, refrigerated storage with EDTA or sodium fluoride can prevent MetHb variations, as can storage without any preservative at room or refrigerated temperature. These preservative/temperature combinations all limit the MetHb modifications during storage after five opening/closing cycles. The presence or absence of preservatives also seems to be influenced by the post-mortem delay. However, as the case number for the different categories of post-mortem delays ranged from n = 4 to n = 6, more investigations should be done to confirm this trend.
The best compromise, considering all of the post-mortem delays, seems to be a refrigerated storage of EDTA-treated blood, for which no significant MetHb variations were observed over four weeks.

Temperature and storage delay

This parameter appears to be the most important, whereas the storage conditions of samples before analysis are not always documented or available. It should also be interpreted considering the presence/absence of preservatives and, in some studies, with concern for the presence of cryoprotectant for in frozen storage.
The major point for this parameter is that simple frozen storage at -20°C should be completely avoided if MetHb measurement is scheduled for later, regardless of the presence or absence of preservatives. Some studies have already observed this result. Hb is converted into MetHb via autoxidation after storage at -20°C

for one week or longer. The magnitude of autoxidation seems to be lower at temperatures colder than -20°C [34]. The MetHb concentrations were practically stable at -80° C or -196°C, regardless of the initial values, for 30 days [32,33] or even longer [35], although considerable autoxidation was observed at -30°C, especially in blood containing small amounts of MetHb [33]. However, this autoxidation could be prevented by adding a cryoprotectant to whole blood. As result, storage with added cryoprotectant at -30°C or without any additions at -80°C or -196°C have proven to be suitable for the long-term storage of blood samples from autopsy cadavers for MetHb determination [33]. However, these results were obtained from blood samples that were fortified with MetHb in vitro by heating or the direct addition of nitrites, leading to potential instability during storage compared with in vivo MetHb. Salt addition can also minimize Hb oxidation during the stress of freeze-thaw operations [36]. Other works have shown that storage ranging from -12 to -20°C presents further problems and should be avoided because the MetHb levels in frozen-thawed specimens increased over time, from 1.8 % at 6 hours to 10.9 % after 6 days [36,37].

An alternative to prevent MetHb increase could be refrigerated storage, but this solution should be limited to short-term storage. Indeed, examples in the literature have shown that MetHb in nonfrozen (refrigerated 1-4°C and room temperature 22-24°C) venous blood samples from a healthy volunteer, as analyzed by CO-oximetry, never exceeded 0.8 % after six days [37]. Other results obtained on blood hemolysates (but not whole blood) showed that dilution in phosphate buffer (pH 7.0) was able to stabilize MetHb for up to 9 days [31]. Our results, which were obtained over four weeks, are in agreement with this trend because neither refrigerated nor room temperature storage led to significant differences in MetHb. The postmortem delay may explain some potential MetHb variations.

However, frozen storage should be preferred in cases of delayed analysis, especially if the sample is suspected to contain high MetHb saturation. Indeed, MetHb is rapidly reduced to Hb by intraerythrocyte MetHb reductase when blood samples are stored as whole blood without freezing [35] . This could lead to an underestimation of MetHb.

Consequently, MetHb analysis should be done as soon as the samples are received at the laboratory. If a sample must be stored, for short-term storage (one or two weeks), room temperature or refrigerated storage in tubes with EDTA is preferred. For longer storage (more than two weeks), frozen storage with cryoprotectant at -80°C or, even better, -196°C should be used (with or without EDTA).

Relevance of post-mortem MetHb

This preliminary storage study aims to identify storage conditions to prevent MetHb changes and investigate the validity of post-mortem MetHb based on reliable measurements. However, the literature is scarce concerning post-mortem MetHb validity. Random post-mortem MetHb variations were observed, and the consensus of the studies' conclusions was the unsuitability of using post-mortem MetHb as an indicator. However, the available studies on this topic used non-optimized storage conditions and were occasionally insufficiently detailed concerning blood sample storage. Indeed, the storage conditions can easily explain the decrease of MetHb in positive samples or its increase in negative samples.

The first study on a post-mortem decrease in MetHb was published in 1974 and stated that the intracellular enzymatic reduction of MetHb to Hb by MetHb reductase could continue after death; enzymatic activity was not effectively inhibited by fluoride but was thermally deactivated (heating during fire) [38]. Similarly, a MetHb decrease was noticed in hemolyzed post-mortem blood due to bacterial contamination [38]. These results are still valid and very informative for defining storage guidelines: post-mortem MetHb should not be measured on contaminated samples, and the analysis should be performed rapidly after collection; if it is delayed, MetHb reductase should be inhibited.

Another study on post-mortem MetHb validity was published in 1984 [24] and concluded that the post-mortem MetHb concentrations are not valid indicators of antemortem methemoglobinemia, especially because of the random MetHb increases observed in negative samples. Cardiac blood from 49 autopsies, with post-mortem delays ranging from 2 hours to 3 days and without evidence of exposure to MetHb-

inducing agents, were measured using an IL CO-Oximeter. They measured a wide range of MetHb saturation levels (from 0.8 to 57 %) and found no relationships between post-mortem delay and MetHb variations. However, the storage conditions could explain these results because the blood samples were collected in tubes with sodium fluoride, immediately frozen, and finally maintained at 0 to -4°C until analysis. No indication was available concerning the storage delay from the sampling to the analysis. Considering the results obtained later [32-34,36,37,39] and those obtained herein, an eventual reason for the MetHb variation could be an autoxidation of hemolyzed hemoglobin caused by freezing the samples, as supported by other scientists [26].

Four years ago, another study was performed on post-mortem MetHb comparing dead victims of exhaust-fume poisoning and fire to dead victims of gas poisoning [25]. The post-mortem MetHb was measured in cardiac blood within 48 h after death and without prior freezing by using three methods (Hitachi 139 Spectrophotometer, a polarographic oxygen analyzer and a cyanmethemoglobin method). The post-mortem MetHb measured in the blood of the victims of fire and exhaust-fume poisoning was found to be significantly higher than that of victims of gas poisoning. Considering the storage conditions, which did not promote an increase in MetHb, these results support the validity of post-mortem MetHb measurement. This conclusion has also been supported by more recent works. A study performed in 2009 on femoral blood obtained from a dead victim suspected of fatal and deliberate automobile exhaust inhalation led to significantly high MetHb saturation (56 %) [26]. The post-mortem delay until the deceased was found was estimated to be several hours, and the story of the body was well documented. From the scene, the body was stored in a refrigerated room for two days, transferred to a university hospital and kept in a refrigerated room (4-6°C) for three days until autopsy (5.5 days after death). A blood sample was collected in a heparinized tube containing potassium fluoride as a preservative, sent by normal post (temperature above freezing), received 3.5 days later and stored at refrigerated temperature until analysis 8 days later (17.5 days after death). Considering the storage conditions, the post-mortem MetHb measurement was considered valid, even

if more reliable results would have been obtained if the analysis had not been delayed and the autopsy had been performed earlier.

The relevance of postmortem MetHb measurement is still under discussion. However, the postmortem MetHb should only be investigated in samples whose sampling and storage are known to not promote MetHb changes.

Limitations

This study aims to determine the best storage conditions for MetHb in blood samples. This work was done with four categories of PMI and a lot of storage variables but the number of cases per category needs to be increased for a higher degree of statistical significance. Once identified, the best storage conditions should be applied to a more important number of postmortem blood samples because the post-mortem delay, cause and circumstances of death could cause initial increases in MetHb.

Conclusion

To avoid MetHb changes, the post-mortem storage delay and delay in sampling for analysis should be reduced as much as possible. If the analysis is delayed for a short time (two weeks), the blood sample taken at autopsy should not be frozen but collected in EDTA preservative and stored under refrigeration (4-6°C) until analysis. If the analysis is delayed for a longer period (more than two weeks), the blood sample should be frozen with cryoprotectant at -80°C or -196°C. Finally, opening/closing cycles should be avoided because they cause potential autoxidation increases. However, blood samples of poor quality due to delays and/or sample heating or putrefaction should not be analyzed because the blood state can cause optical artifacts for CO-oximetry or spectrophotometry measurements, leading to MetHb misestimations. If blood storage

conditions are not sufficiently precise, post-mortem MetHb should be done simultaneously with blood and urine nitrate/nitrite measurements. MetHb variations that are not correlated to nitrate/nitrite variations should be carefully interpreted because an increase in MetHb can come from autoxidation, and a MetHb decrease can come from MetHb reductase activity.

Considering these storage guidelines, the post-mortem MetHb should be widely monitored to confirm the validity of using the post-mortem MetHb as a methemoglobinemia indicator.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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List of figures

Figure 1. MetHb measurements (%) obtained from blood sampled from bodies with post-mortem delay < 12 hours.

D: Day; CB: Cardiac Blood and PB: Peripheral Blood

NaF: Sodium fluoride; EDTA: Ethylenediaminetetraacetic acid; Nat : Native blood / no preservative

Figure 2. MetHb measurements (%) obtained from blood sampled from bodies with post-mortem delay between 12 and 24 hours.

D: Day; CB: Cardiac Blood and PB: Peripheral Blood

NaF: Sodium fluoride; EDTA: Ethylenediaminetetraacetic acid; Nat : Native blood / no preservative

Figure 3. MetHb measurements (%) obtained from blood sampled from bodies with post-mortem delay between 24 and 48 hours.

D: Day; CB: Cardiac Blood and PB: Peripheral Blood

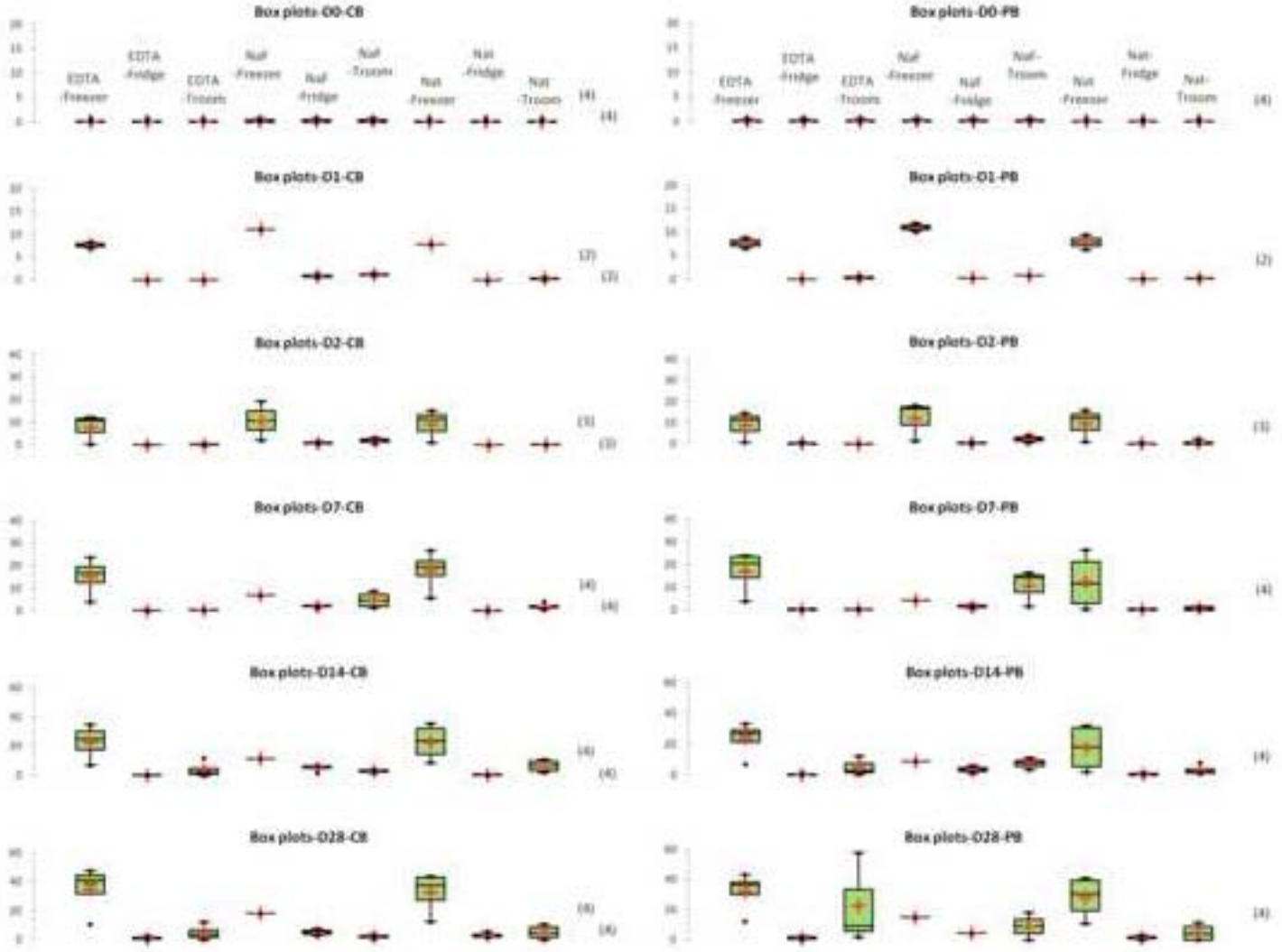
NaF: Sodium fluoride; EDTA: Ethylenediaminetetraacetic acid; Nat : Native blood / no preservative

Figure 4. MetHb measurements (%) obtained from blood sampled from bodies with post-mortem delay > 48 hours.

D: Day; CB: Cardiac Blood and PB: Peripheral Blood

NaF: Sodium fluoride; EDTA: Ethylenediaminetetraacetic acid; Nat : Native blood / no preservative

Figure 1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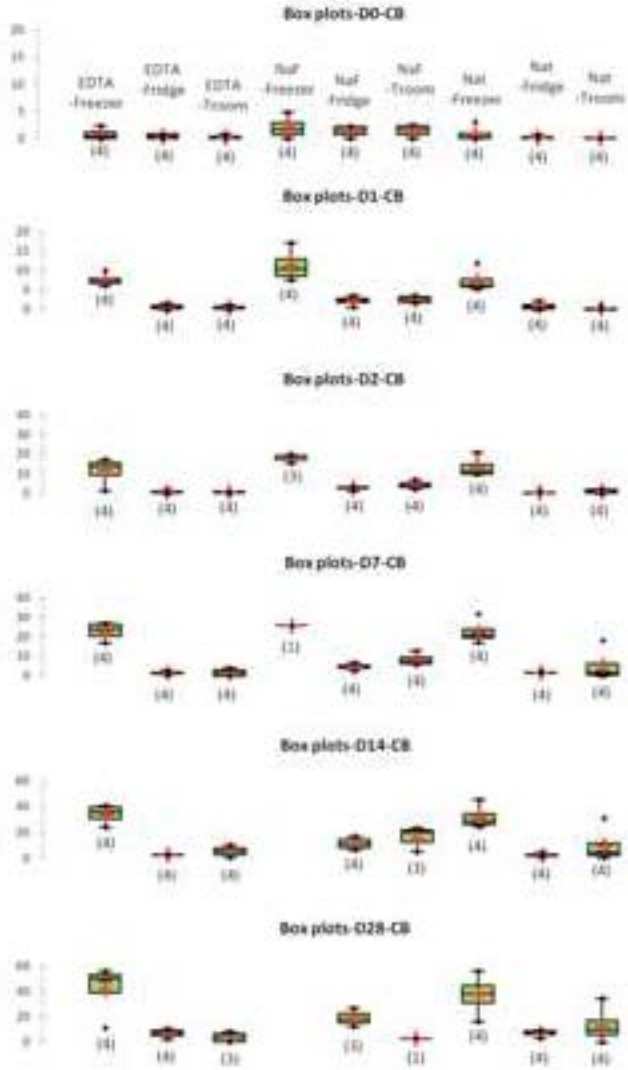
Box plots-D0-PB
Box plots-D1-CB
Box plots-D1-PB
Box plots-D2-CB
Box plots-D2-PB
Box plots-D7-CB
Box plots-D7-PB
Box plots-D14-CB
Box plots-D14-PB
Box plots-D28-CB
Box plots-D28-PB
EDTA Freezer
EDTA Fridge
EDTA Troom
Nat Freezer
Nat Fridge
Nat Troom

Figure 2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EDTA -Troom
NaF -Freezer
NaF -Fridge
NaF -Troom
Nat -Freezer
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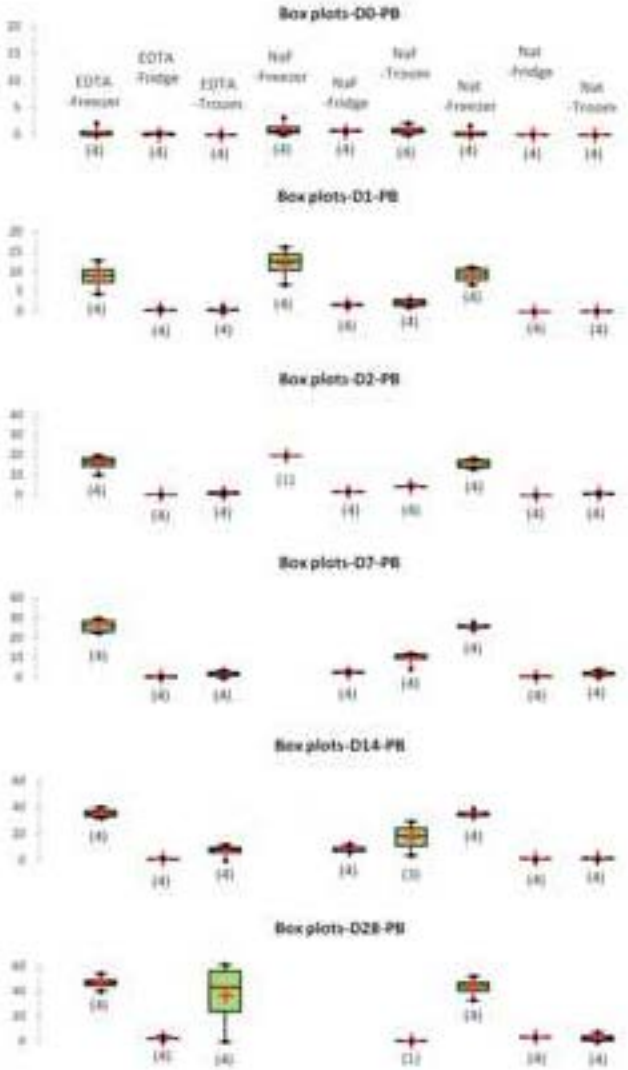
Nat -Troom
Box plots-D1-CB
Box plots-D2-CB
Box plots-D7-CB
Box plots-D14-CB
Box plots-D28-CB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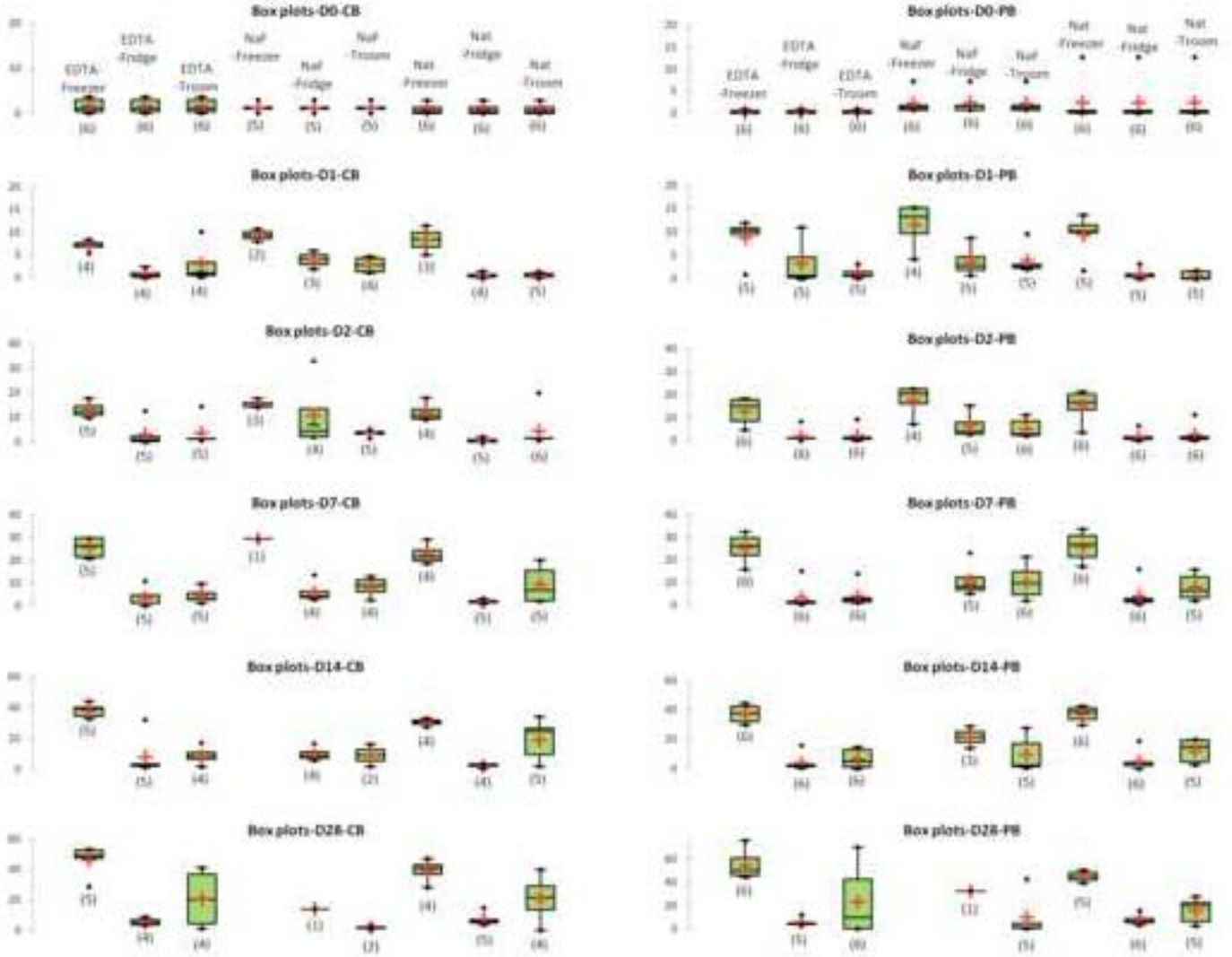
NaF -Fridge
NaF -Troom
Nat -Freezer
Nat -Fridge
Nat -Troom
Box plots-D1-PB
Box plots-D2-PB
Box plots-D7-PB
Box plots-D14-PB
Box plots-D28-PB

Figure 3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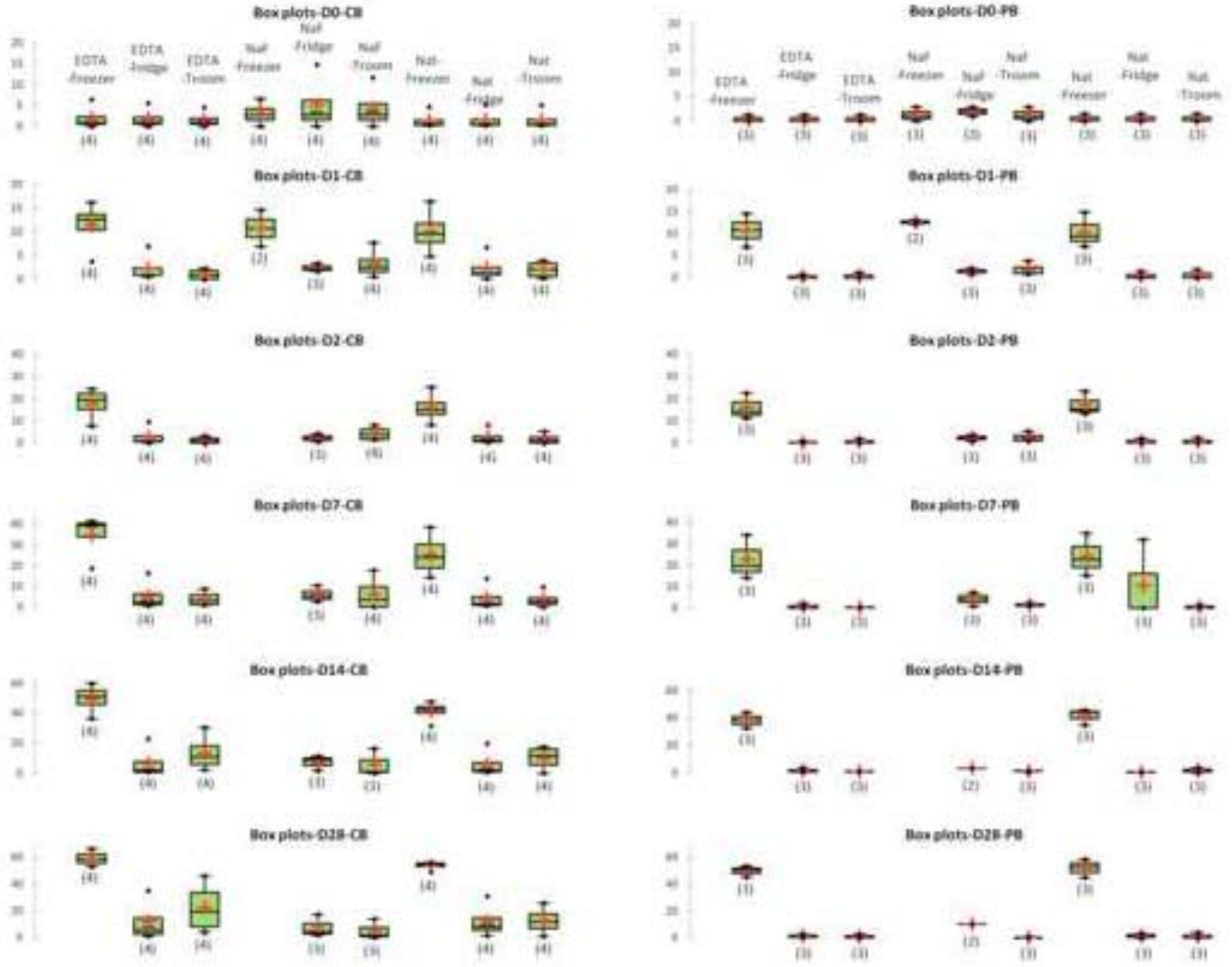
Box plots-D14-PB
Box plots-D28-CB
Box plots-D28-PB

Figure 4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Box plots-D7-C8
Box plots-D7-P8
Box plots-D14-C8
Box plots-D14-P8
Box plots-D28-C8
Box plots-D28-P8

Table 1

Table 1. P-values results of multifactorial analysis of variance (ANOVA) performed on MetHb measurements at various storage conditions and delays

Day	PM	Blood	Preservative	Temperature	PM × Blood	PM × Preservative	PM × Temperature	Blood × Preservative	Blood × Temperature	Preservative × Temperature	PM × Blood × Preservative	PM × Blood × Temperature	PM × Preservative × Temperature	Blood × Preservative × Temperature
D0	< 0.0001	0.164	0.006	0.953	0.011	0.249	0.964	0.156	0.973	0.997	0.187	1.000	1.000	0.998
D1	0.230	0.487	0.001	< 0.0001	0.310	0.935	0.002	0.912	0.547	0.995	1.000	0.957	0.938	0.969
D2	< 0.0001	0.491	0.206	< 0.0001	0.594	0.693	0.006	0.753	0.218	0.620	0.990	0.996	0.201	0.904
D7	< 0.0001	0.445	0.005	< 0.0001	0.181	0.544	0.000	0.685	0.860	0.045	0.380	0.581	0.352	0.659
D14	< 0.0001	0.056	< 0.0001	< 0.0001	0.304	0.017	< 0.0001	0.706	0.263	0.021	0.603	0.592	0.361	0.354
D28	0.001	0.943	< 0.0001	< 0.0001	0.023	0.952	0.013	0.370	0.427	0.003	0.435	0.485	0.819	0.375

PM: post-mortem delay; In gray: parameters or interactions between parameters that did not significantly influence MetHb variations during storage.
ANOVA performed with a p-value of 0.05 %