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Stability of postmortem methemoglobin: Artifactual changes caused by storage conditions

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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.

1. 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].

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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.

2. Materials and methods

2.1. 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 h (n = 4), 12 < PMI < 24 h (n = 4), 24 < PMI < 48 h (n = 6) and PMI > 48 h (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 h are very rare, as are analyzable samples coming from cases with PMI > 48 h.

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.

2.2. 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.

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%.