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Method of Stabilizing Blood for the Determination of Methemoglobin

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Methemoglobin (MetHb) is a significant clinical problem for some poisonings. Its measurement is a problem as both formation and reduction of MetHb can occur even after sampling with time. The objective of this study was to discover a method to stabilize the blood samples for the determination of MetHb. First, hemolysates were prepared by diluting the MetHb blood samples with phosphate buffers under different pH values. The samples were stored at 4–8°C and a day-to-day variability in the amount of MetHb was determined using the method described by Evelyn and Malloy. The results show that there is a significant change in the amount of MetHb stored in both KH_2PO_4/Na_2HPO_4 and $KH_2PO_4/Na_2HPO_4.2H_2O$ buffer solutions at pH of 6.7 and 6.9. Buffer solution containing phosphate composition of $KH_2PO_4/Na_2HPO_4 \cdot 2H_2O$ (pH = 7.0) gives relatively stable values for MetHb during the storage and the amount of MetHb samples in the buffer solution retain constant up to 9 days. Therefore, stabilized MetHb blood samples can be prepared using $KH_2PO_4/Na_2HPO_4 \cdot 2H_2O$ buffer solution (pH = 7) with non-ionic detergent and the samples can be stored for several days at 4–8°C. J. Clin. Lab. Anal. 25:366–368, 2011.

Key words: methemoglobin; blood storage; stability; spectrophotometry; toxicology

BACKGROUND

Methemoglobin (MetHb) is hemoglobin that has been oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state, thus unable to bind oxygen. The NADH-MetHb reductase enzyme reduces MetHb to hemoglobin in our body. Methemoglobinemia results from either inadequate enzyme activity or too much MetHb production.

MetHb is continuously being formed in the normal red blood cells by the process of autooxidation of ferrous iron of the haem complex to the ferric form. At the same time, MetHb is rapidly reduced to hemoglobin by intraerythrocytic MetHb reductase after sampling. Once collected from the patient, blood MetHb levels increase with time. MetHb production is a serious life-threatening consequence of exposure to a number of toxins, especially in the developing world. The efficacy of antidotes in an individual patient is assessed by measuring MetHb false elevations from postsampling changes and can be interpreted as antidote failure and influence subsequent clinical care. Owing to the instability of the MetHb in the drawn blood sample, the usual clinical recommendation is to carry out the assay as rapidly as possible using a fresh sample (1). The spectroscopic method is the analytical method of choice for determination of MetHb as co-oximetry and pulse oximetry illustrate false elevation after methylene blue therapy (2). In this article, a very simple new method is described for stabilization of MetHb for the determination of MetHb levels.

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MATERIAL AND EXPERIMENTAL METHOD

Reagents

Sodium chloride, sodium phosphate dibasic dehydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium phosphate dibasic (Na_2HPO_4), and potassium orthophosphate (KH_2PO_4) were obtained from Sigma chemicals (Fluka, Germany). Triton-X 100 was used as the detergent. Heparinized venous blood samples obtained from hospital patients were provided by the Laboratory of Peradeniya Teaching Hospital, after necessary determination of MetHb were done.

TABLE 1. Stability of MetHb% as Whole Blood and Buffer Solution in Freezer (−20° C)

MetHb %	Time/days	Whole blood	Haemolysate
21.4%	1	28.9	27.2
	2	40.3	38.5
	3	55.9	45.9
30.2%	1	37.5	38.1
	2	46.7	45.5
	3	55.5	51.3
42.1%	1	48.3	47.5
	2	55.0	52.4
	3	63.1	59.2
51.5%	1	57.3	57.0
	2	65.0	61.4
	3	72.1	68.3

Preparation of Reagents and Hemolysate

A 0.1 mmol solution of $Na_2HPO_4 \cdot 2H_2O$ and Na_2HPO_4 were prepared in deionized water. The pH was adjusted with 0.1 mmol solution of KH_2PO_4. The following combinations of phosphate buffers KH_2PO_4/Na_2HPO_4, $KH_2PO_4/Na_2HPO_4 \cdot 2H_2O$ were prepared using the standard solutions to make pH = 6.7, 6.9, and 7.0 buffers.

MetHb (from 10 to 100%) blood samples were prepared by treating normal blood with $NaNO_2$ (3). The blood sample's MetHb concentration was measured using the method described by Evelyn and Malloy (4). Hemolysates were prepared by adding 0.2 ml of the MetHb blood sample to 10.0 ml of MetHb stabilizing solution containing phosphate buffers and non-ionic detergent in different pH.

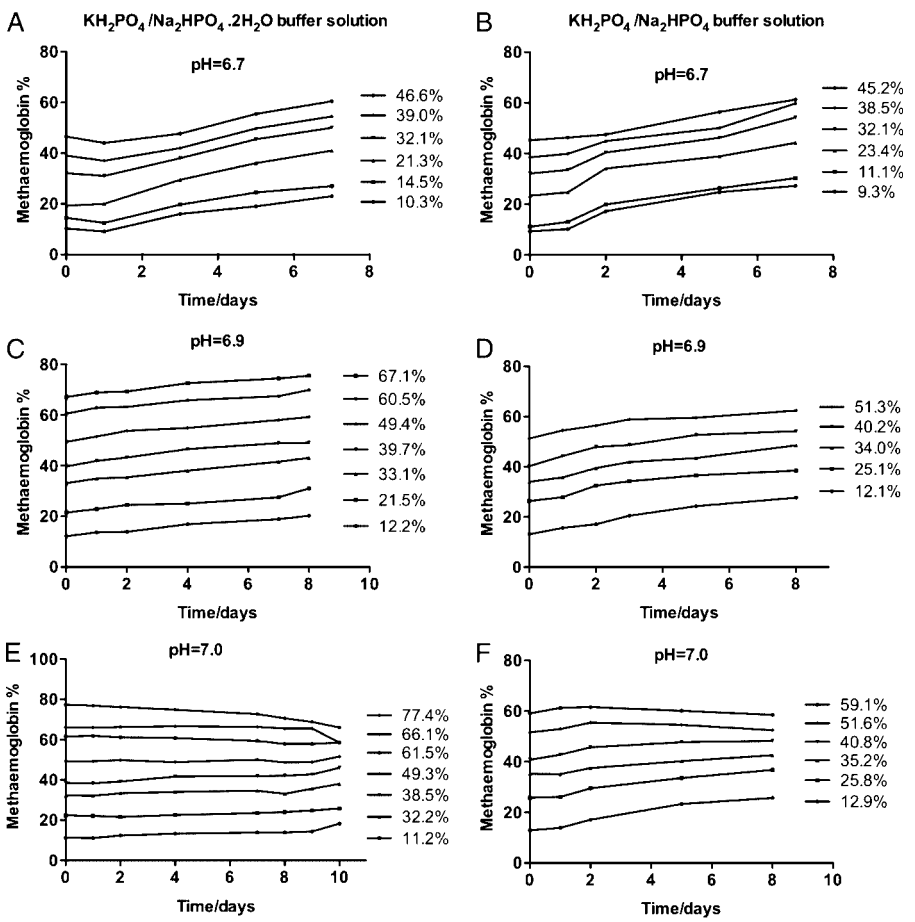

Fig. 1. Stability of MetHb blood in buffers at different pH levels stored at 4–8°C. **A, C, E:** MetHb hemolysates in $KH_2PO_4/Na_2HPO_4 \cdot 2H_2O$ buffer. **B, D, F:** MetHb hemolysates in KH_2PO_4/Na_2HPO_4 buffer.

Storage of MetHb Blood Samples

The MetHb buffers were stored at 4–8°C in a refrigerator. A day-to-day variability in absorbance was measured at a wavelength of 630 nm using a spectrophotometer and the amount of MetHb was determined.

RESULTS

When blood samples were stored at 0–20°C, considerable autoxidation occurred at both whole blood and the hemolysate (Table 1). Figure 1A and B show that the amount of MetHb in both KH_2PO_4/Na_2HPO_4 and $KH_2PO_4/Na_2HPO_4 \cdot 2H_2O$ buffer solution increases rapidly up to the second day and gradually increases thereafter at pH of 6.7. Whereas at pH = 6.9 (C and D) the increase in MetHb decreases in both buffers. Buffer solution containing phosphate composition of $KH_2PO_4/Na_2HPO_4.2H_2O$ (pH = 7.0) (Fig. 1E and F) gives relatively stable values for MetHb during the storage. Figure 1E shows that the amount of MetHb samples in the buffer solution remain constant for up to 9 days. However, slight autoxidation was observed 9 days after storage. These results suggest that MetHb reductase preventing autooxidation was nearly inactivated by refrigerating. Present results show that the storage of MetHb hemolysate at refrigerated temperature works well and practically stable until at least 9 days.

DISCUSSION

The stability of MetHb as whole blood at room temperature or refrigerated at or below −20°C results in the oxidation hemoglobin to MetHb (1). The modification of hemoglobin structure induced in the frozen state at −20°C may have caused oxidation of hemoglobin iron, and that the oxidation was greatly inhibited. Autooxidation of the sample may occur if it is left at warmer temperatures, especially in samples with low MetHb levels (5). Several researchers reported that MetHb became unstable faster at alkaline pH values than at acid pH values (6). Phosphate anions, such as many other anions, can decrease the oxygen affinity of hemoglobin (7). The buffer serves to fix the valence state of the iron in heme at a concentration which is representative of the system in a blood sample at the time of collection, and maintain that fixed state for an extended period of time. The buffer solution, which may contain an amount of MetHb, is stored in a container that is resistant to oxygen and prevents autoreduction of any MetHb to proceed. Even more research on the stability has been done using NADPH, storing at −190°C. This method is easier to determine the MetHb using the Evelyn and Malloy method. According to this technique, the level of MetHb is easily determined in a blood sample by mixing the blood with a buffer composition, which stabilizes the relative concentrations of ferrous and ferric hemoglobin forms at pH = 7 for accurate measurements for MetHb. The time between sample collection and spectrophotometric analysis can be conveniently increased using a buffer solution, which stabilizes MetHb and prevent further MetHb production.

CONCLUSION

A stabilized MetHb blood sample can be prepared using $KH_2PO_4/Na_2HPO_4.2H_2O$ buffer solution with non-ionic detergent at pH = 7, and the samples can be stored for 9 days at 4–8°C.

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