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References

Addition of Sodium Fluoride to Whole Blood Does Not Stabilize Plasma Homocysteine But Produces Dilution Effects on Plasma Constituents and Hematocrit, Martin Patrick Hughes,† Timothy H. Carlson,† M. Kathleen McLaughlin,† and Daniel D. Bankson 1,2 (1 Department of Laboratory Medicine, University of Washington, Seattle, WA 98195 and 2 Veterans Affairs Puget Sound Health Care System, Seattle, WA 98108; † present address: Pacifi Inc., Seattle, WA 98119)

Interest in total plasma homocysteine (Hcy) measurements has increased with the availability of evidence that even mild hyperhomocysteinemia is an independent risk factor for cardiovascular disease (1). Consequently, the variability of current Hcy results has been the subject of considerable research (2–4). Whole blood stored at room temperature after phlebotomy shows an increase in Hcy concentrations of ~10% per hour, apparently because of Hcy synthesis and release from erythrocytes (5, 6). Previous studies have shown that the best method for avoiding falsely high Hcy results is prompt postphlebotomy centrifugation and separation of the plasma from the red blood cells (6) or storage of whole blood samples at 0°C if prompt centrifugation is not feasible (3). The use of anticoagulants as preservatives in whole blood has also been advocated (2–4); among these, sodium fluoride (NaF) in particular has been described as useful when added to whole blood at suggested concentrations of up to 4 g/L (95 mmol/L) (4, 7).

Many laboratories, therefore, now use NaF as a preservative in the blood collection tubes used for Hcy assays. We measured plasma Hcy, using a modified version of the HPLC method with fluorescence detection introduced by Refsum and co-workers (8), after the addition of pure NaF (2.5 g/L, 60 mmol/L) to heparinized whole blood. The studies were approved by the University of Washington Institutional Review Board and involved the donation of blood from men and women after informed consent. We found minimal inhibition of the time-dependent increase in Hcy concentrations. We also investigated the preservative abilities of Na2EDTA (1.5 g/L, 4.0 mmol/L), EGTA (1.5 g/L, 3.9 mmol/L), and thymol (1.0 g/L, 6.6 mmol/L), again added in pure form to heparinized whole blood. All chemicals were obtained from the Aldrich Chemical Co. and used without further purification. None provided satisfactory results. For example, during 8 h at 25°C, plasma Hcy increased as follows: control (containing no preservative), 8.9% per hour; thymol, 9.8% per hour; EGTA, 8.0% per hour; Na2EDTA, 6.5% per hour; and NaF, 6.0% per hour.

During the studies of these additives, we were intrigued to observe a decrease in the Hcy concentrations of the NaF-containing samples at all time points as compared with the blank control and compared with the samples that contained the other potential preservatives. During further investigations, we discovered that addition of NaF to heparinized whole blood caused an immediate NaF concentration-dependent decrease in spun hematocrit (Table 1). This led us to propose that on addition to the whole blood sample, the NaF produced hypertonic plasma with subsequent desiccation of the red blood cells. The resulting osmosis effectively diluted the plasma to such an extent that it lowered the apparent Hcy concentration by ~10% at 100 mmol/L NaF. Plasma albumin (measured by bromcresol green on the Paramax RX analyzer) also decreased linearly with increasing concentrations of NaF, confirming that the change in hematocrit was not artifactual (Table 1). Because lithium is not excluded from the erythrocyte and so does not cause the same concentration-dependent plasma dilution, we investigated the preservative properties of lithium fluoride (LiF, Fisher Scientific). LiF decreased hematocrit, albumin, and Hcy <3% (Table 1).

Next, the time-dependent effect of LiF and NaF on plasma Hcy was investigated. Whole blood with K3EDTA anticoagulant (1.8 g/L) was transferred into sample tubes containing dry LiF, dry NaF, or no preservative. These salts (50 mmol/L blood) both inhibited the rise of Hcy concentrations at 25°C only minimally, compared with a control with no additive (Fig. 1). In fact, storage of the blood samples on ice provided the only effective inhibition of plasma Hcy increase over time. It is interesting to note that the plasma Hcy concentration of the NaF-
preserved samples at a time of ∼2.5 h is essentially identical to the value of the control sample processed immediately after phlebotomy (time, 0 h, Fig. 1). These results indicate that the artifactual negative dilution effect of NaF completely offsets the positive Hcy-production effect in these samples after 2–3 h. These data might be improperly interpreted as a true Hcy preservative effect.

We reviewed the report of the apparent Hcy-preservative effect of NaF. When this salt was used as an anticoagulant (60 mmol/L, 2.5 g/L), the increase in plasma Hcy was <30% at 24 h, compared with an increase of >180% for samples containing 4.4 mmol/L EDTA (1.6 g/L) as anticoagulant (2). Although this indicated that NaF could not completely prevent the plasma Hcy increase, its superiority to EDTA led to studies of its preservative ability. These studies eventually led to the recommendation that relatively high concentrations of NaF (4 g/L, ∼95 mmol/L) be used as a preservative in Hcy measurements (4, 7).

NaF has long been used in blood glucose measurements as an inhibitor of glycolysis. An effect of NaF on Hcy release by erythrocytes might, in theory, be attributable to its inhibition of glycolysis, and the resulting inhibition of ATP production. In the homocysteine pathway, methionine (Met) is converted to S-adenosylmethionine (SAM) in an ATP-dependent step. Conversion of SAM to S-adenosylhomocysteine and finally hydrolysis to homocysteine (9) follow this. Although lack of ATP would inhibit the initial step of this pathway (conversion of Met to SAM), our studies suggest that there may be sufficient cellular reserves of SAM and/or S-adenosylhomocysteine to provide continued production of Hcy despite inhibition of ATP synthesis. This would explain why other compounds, including thymol and other potential inhibitors of glycolysis such as LiF, EDTA, and EGTA, are inefficient as Hcy preservatives.

For example, the LiF-containing samples incubated at 25 °C showed a slight inhibition of the rise in plasma Hcy concentrations over time compared with the control samples at the same temperature (Fig. 1). This modest improvement could be a result of the superior ability of LiF to directly inhibit glycolysis (and consequently the production of ATP), thus indirectly inhibiting the conversion of Met to SAM. However, the ideal preservative must also inhibit the enzymatic steps downstream in the Hcy cycle, something which simple glycolytic inhibitors apparently cannot accomplish. This broad enzymatic inhibition is achieved to an appreciable extent, however, by storage of the whole blood samples at 0 °C. We therefore conclude that the most reliable method of blood preservation for Hcy measurements is rapid cooling of the samples on wet ice. Even slightly higher storage temperatures (4 °C) have proven ineffective in preventing Hcy increases (2, 6).

We confirm previous reports of the ineffectiveness of NaF in preventing false increases in plasma Hcy measurement (2). We have also shown that the use of NaF in fact leads to dilution errors, affecting several plasma analyte concentrations, including albumin and Hcy. This dilution
effect may confound studies, planned or already completed, in which these analytes are measured. We therefore recommend that NaF not be used as a preservative in Hcy measurements. Other preservatives studied to date have proven ineffective or problematic. A recent report suggests that acidified citrate at pH 4.3 be considered as a whole blood preservative because it inhibits the rise in Hcy over time to a degree similar to storage on crushed ice (10). However, the authors note that an added complication in this case was that the Hcy concentrations at each time point were falsely increased (by ~10%) for the citrate samples compared with controls stored at 0 °C in EDTA-containing tubes. We believe that storage of blood samples on wet ice after phlebotomy, along with rapid centrifugation and separation of the plasma, are the only means of providing accurate Hcy measurements shown to date.

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References

Verification of Multichannel Liquid Dispenser Performance in the 4–30 μL Range by Using Optical Pathlength Measurements in Microplates, Evelyn L. McGown,* Kirk Schroeder, and Dean G. Hafeman (Molecular Devices Corporation, 1311 Orleans Drive, Sunnyvale, CA 94089; *author for correspondence: fax 408-747-3602, e-mail evelyn_mcgown@moldev.com)

Pipettors and liquid handling systems should be checked regularly to verify their precision and accuracy. Professional organizations recommend performing 4-replicate tests monthly and 10-replicate tests at least quarterly (1–4). Traditional gravimetric procedures are adequate to meet these testing requirements for single-channel pipetors but are tedious and impractical for multichannel devices. An alternative is to dispense a solution of colored dye into wells of a microplate and to measure the resulting absorbance values in a microplate reader. Volume calibration is performed with a calibration curve relating absorbance to volume (5).

We recently reported a method for verifying multichannel pipettor performance by a spectrophotometric procedure that utilizes the near infrared absorbance of water and does not require addition of a dye (6). Water or other aqueous reagent is dispensed from the pipettor into microplate wells, and the optical pathlength in each well is determined in a microplate spectrophotometer. Water is essentially transparent from 200 to 900 nm but has a distinctive absorbance peak near 977 nm. As predicted by the Lambert law of light absorption, absorbance is proportional to the distance that light travels through the sample; thus the characteristic absorbance of water can be utilized to measure the pathlength of an aqueous sample. The maximum absorbance is affected by temperature; however, temperature dependency can be avoided by making the absorbance measurements at a temperature isosbestic point (near 1000 nm). Baseline absorbance is measured at a wavelength distant from the water absorbance peak, e.g., 900 nm, where again the absorbance is independent of temperature. The pathlength through an aqueous reagent in a microplate well is calculated from the difference between peak and baseline absorbance in the well and the value obtained by making the same measurements on the reagent in a standard 1-cm cuvette:

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\frac{(A_{1000} - A_{900}) \text{ Reagent in Well}}{(A_{1000} - A_{900}) \text{ Reagent in 1-cm Cuvette}} = \text{Pathlength in Well (cm)}
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In the published procedure, the lower end of the range of dispense volumes (30 μL) is limited by variable meniscus formation in nearly empty wells. We have now devised a modification of that method to extend the range to much lower volumes. By using half-area microplates and an incremental pipetting method, dispense volumes of 4 μL or less can be accommodated.

The inset in Fig. 1 illustrates the principle of the method. The first step is to put sufficient aqueous reagent into a microplate well to cover the bottom and to establish a uniform meniscus. A measurement of the initial optical pathlength (P1) is made. Without delay, the aqueous dispense volume is pipetted, and a second measurement of the optical pathlength (P2) is made. The difference between P2 and P1 is the pathlength increment associated with the dispense volume pipetted. In the example presented below, the initial volume of water was 25–40 μL, and subsequent dispense volumes pipetted were 1.0–30 μL. Half-area microplates (Corning Costar Corp.) were used to maximize sensitivity. All absorbance measurements (at 900 nm and 1000 nm) for pathlength calcula-