Effects of Temperature on Stability of Blood Homocysteine in Collection Tubes Containing 3-Deazaadenosine

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Background: The accuracy of homocysteine (Hcy) results is currently compromised by the requirement to separate the plasma within 1 h of sample collection. We studied the effect of temperature on the stability of plasma Hcy over a 72-h time course in blood collected into evacuated tubes containing either EDTA alone or both EDTA and 3-deazaadenosine (3DA).

Methods: We recruited 100 volunteers, including both diseased and healthy individuals with a range of baseline plasma Hcy values, from two centers. Blood samples were collected into tubes containing EDTA, and EDTA plus 3DA and stored at ambient temperature (20–25 °C) or refrigerated (2–8 °C). Aliquots of blood were centrifuged at various times up to 72 h, the plasma was removed, and Hcy was measured by HPLC.

Results: Plasma Hcy measurement covering the sample collection and storage conditions during the whole time course was possible on samples from 59 of those recruited. One-way ANOVA for repeated measures within subjects revealed that only samples that were collected into tubes containing EDTA plus 3DA and stored refrigerated were stable over 72 h (P = 0.2761).

Conclusions: A combination of 3DA and storage at 2–8 °C will allow collection of samples for plasma Hcy measurement outside of the hospital setting and wider population screening.

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Homocysteine (Hcy), usually present in small amounts in plasma, is formed in mammals solely from methionine (1). McCully and Ragsdale (2, 3) highlighted the importance of this pathway when they found that marked increases in plasma Hcy are a common factor in the presence of vascular lesions, which were brought about either by enzyme deficiencies in methionine metabolism or experimentally in rabbits. Since these initial observations were reported, links between hyperhomocysteinemia and a multitude of disorders have been postulated, including cancer (4), neural tube defects (5), cognitive decline (6), and dementia (7). However, there is still argument about whether increased Hcy is the cause or result of tissue damage (8). Those studies exhibiting a link between Hcy and vascular disease tend to show that relatively small changes in Hcy concentration lead to large increases in relative risk. In a metaanalysis of 27 such studies (9), a 5 μmol/L Hcy increase led to odds ratios for coronary artery disease of 1.6 (95% confidence interval, 1.4–1.7) in men and 1.8 (95% confidence interval, 1.3–1.9) in women. Dietary fortification with folic acid supplements has been shown to decrease Hcy concentrations (10–12), but the results of prospective studies are needed to show whether lowering Hcy is beneficial in reducing the risk of disease (13–15). If confirmed, then the debate is strengthened in favor of screening programs designed to lower the incidence of heart disease. Such moves have been facilitated by the recent emergence of commercial systems allowing the routine clinical laboratory to perform testing previously performed only in research facilities.

The remaining problem is how to stabilize plasma Hcy concentrations in blood before processing, centrifugation, and storage. Erythrocyte Hcy concentrations are ~10-fold
lower than in plasma; therefore, any increase in plasma Hcy after sample collection is not attributable to leakage from the cells, but from continued metabolism and excretion into the plasma (16). The liver and pancreas are mainly responsible for Hcy removal in vivo (1). Because this removal pathway is absent in vitro, Hcy has been shown to increase by as much as 10% per hour over the first few hours after sample collection (16,17). The current recommendation is to place samples on ice and centrifuge within 1 h. Plasma Hcy may then be stable for at least 24 h at room temperature and for several months, if not years, when stored frozen (17).

If a 5 μmol/L increase is associated with an 80% increased risk of vascular disease, artificial increases in plasma Hcy caused by delays in sample processing could easily lead to false-positive results being reported. Although the treatment for hyperhomocysteinemia is simple and noninvasive, proper risk assessment demands accurate data.

Storage temperature (18–21), acid citrate (18, 22–24), sodium fluoride (25, 26), and erythrocyte lysis (27–29) have all been considered in attempts to stabilize plasma Hcy. However, several of these methods cause sample dilution, either through the addition of liquid or, particularly with sodium fluoride, through osmotic effects caused by production of hypertonic plasma (30). To date, the only method that has been shown to stabilize Hcy concentrations in whole blood for any longer than a few hours, without readjustment of reference intervals or immediate centrifugation, is the use of 3-deazaadenosine (3DA) (31). At 100 μmol/L in EDTA whole blood, al Khafaji et al. (31) reported that 3DA stabilized plasma Hcy for 72 h at room temperature before centrifugation. 3DA prevents Hcy production through competitive inhibition of the enzyme S-adenosylhomocysteine hydrolase (SAHH), the final enzyme in Hcy production from methionine.

In pilot studies (32), we investigated the use of 100 μmol/L 3DA in EDTA whole blood before developing a commercially available evacuated blood collection system. However, because 3DA acts through competitive inhibition, its effectiveness is influenced by temperature. To avoid ambiguity when using phrases such as “ambient” temperature, we controlled storage temperatures between 20 and 25 °C; however, the mean Hcy increased from 8.5 μmol/L to 11.5 μmol/L over 72 h. At 2–8 °C, the mean Hcy decreased by a statistically insignificant 0.5 μmol/L.

In light of the pilot study results, a trial batch of evacuated tubes was produced that contained 3DA spray-dried into tubes containing EDTA. Here we report clinical validation of these blood collection tubes, conducted in such a way as to confirm whether a combined effect of low temperature and 3DA was sufficient to provide long-term stability of plasma Hcy in whole blood.

Most studies on sample stability have been conducted on apparently healthy populations. In the clinical validation of our tubes, we were interested in comparing the stability of samples collected from a more diverse population, including patients with vascular disease and elderly patients, in addition to healthy volunteers.

Materials and Methods

Volunteers were recruited in accordance with the current revision of the Helsinki Declaration of 2000 (33). The only exclusion criterion was individuals known to have recently taken drugs that can interfere with the method used for Hcy analysis (captopril, cysteamine, N-acetylcysteine, and N-2-mercaptoethylglycine).

To ensure a range of starting concentrations, two centers were selected such that blood was collected from 50 individuals, patients, and employees at a hospital in the United Kingdom (Birmingham Heartlands Hospital) and from another 50 volunteers, mainly healthy students and employees, at a university campus in the US (University of Maryland at Baltimore). Local approval was obtained from the appropriate ethics committees, and informed consent was given by all participants.

Blood was collected, by venipuncture, into tripotassium EDTA Vacutainer™ Tubes (Becton Dickinson) and DS30 Hcy Blood Collection Tubes (Drew Scientific Ltd., Barrow in Furness, United Kingdom). The DS30 Hcy blood collection tubes contain dipotassium EDTA as anticoagulant and 100 μmol/L 3DA (final concentration) to inhibit Hcy production. Each tube was evacuated to collect 2.5 mL of whole blood. Once collected, each tube was mixed and aliquoted for storage at ambient temperature (20–25 °C) and under refrigeration at 2–8 °C. Samples were taken from each blood tube for baseline Hcy measurements. At 3, 6, 24, and 72 h after blood collection, an aliquot of blood was removed from each tube at each storage temperature and centrifuged for 10 min at 11 000 g. The plasma was removed and stored at −80 °C until analysis.

Hcy measurement was performed at two sites using the Drew DS30 Hcy Analyzer (Drew Scientific). The Drew analyzer uses reversed-phase HPLC with fluorescence detection to separate ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F)-derivatized thiols in plasma. Reduction of disulfide bonds by tris(2-carboxyethyl)phosphine allows measurement of all forms of homocyst(e)ine.

To avoid between-batch variability, each individual’s set of samples was measured within a single run. Within-batch imprecision for this method has been shown to be <2% (34).

Changes in Hcy concentration for each storage condition over time were assessed for statistical significance by one-way ANOVA within subjects with repeated measures.

Results

Of the 100 volunteers recruited, 59 yielded a complete set of results for all time points and storage conditions under investigation. Reasons for lost results included poor
bleeds from elderly volunteers and identifiable mistakes in sample analysis. Incomplete datasets were not included.

The 59 volunteers comprised 38 females and 21 males. The median age was 47 years, with a range of 17–91 years. Initial plasma Hcy values ranged from 3.9 to 28.7 μmol/L.

Shown in Fig. 1 are the changes in plasma Hcy concentration over time. One-way ANOVA within subjects with repeated measures showed that only a combination of the DS30 Hcy blood tube and refrigerated storage provided stability of plasma Hcy concentrations in whole blood at all times up to 72 h (no significant difference, \( P = 0.2761 \)). Hcy changes under the three other storage conditions were all highly significant (\( P < 0.0001 \)).

Using the Tukey \( \omega \)-procedure (35), we assessed the time taken for Hcy values to change significantly from baseline for each condition. In EDTA alone, Hcy increased significantly before 3 h had elapsed, regardless of whether the tubes were refrigerated or left at ambient temperature. However, in DS30 Hcy blood collection tubes, 3DA used in combination with EDTA provided added stability at ambient temperature (6 h).

DS30 Hcy blood tube samples from 17 individuals yielded enough blood to allow analysis of plasma after 1 week (168 h) at 2–8 °C. However, these volunteers did not produce a full dataset for the other storage conditions. Again, no change in Hcy concentration was observed (\( P = 0.4690 \)). The initial mean Hcy for this group was 14.0 μmol/L. Even after 1 week at 2–8 °C, the concentration had increased by only 0.3 μmol/L.

Previous stability studies have reported changes in Hcy over time as either percentages or absolute changes against initial values. In the present study, in the absence of inhibitor, whole-blood samples stored at 20–25 °C showed an average Hcy increase of 8% per hour in the first few hours after sample collection, but those individuals whose Hcy was at the lower end of the reference interval (5.2–15.1 μmol/L (36)) showed increases as large as 20% per hour. Dividing the study population into 5 μmol/L bands (Table 1), according to baseline Hcy, highlighted a significant difference (\( P < 0.001 \)) in the percentage change in plasma Hcy in the whole-blood samples over 72 h across the different bands. When analyzed according to absolute changes in Hcy, this statistical significance disappeared (\( P = 0.1460 \)). This shows that the mass of Hcy produced over time varies little between blood samples taken from a large group of individuals, even over a wide range of initial Hcy concentrations. It therefore follows that the percentage change will be greater in those samples with the lowest starting concentrations.

In light of these findings, we believed that data obtained from individuals with higher starting Hcy might mask any changes over time in those samples with initial Hcy in the lower groups. We therefore subdivided the data into two groups (\( \leq 10 \) μmol/L and \( > 10 \) μmol/L) containing 43 and 16 individuals, respectively, and analyzed them (Table 2). Irrespective of the baseline Hcy, storage of whole blood at 2–8 °C in EDTA or 20–25 °C in the presence or absence of 3DA led to significant changes in plasma Hcy over 72 h (\( P < 0.0001 \)), whereas samples showed stability over 3 days of storage at 2–8 °C in the DS30 Hcy blood tube independent of whether the initial Hcy was less than or greater than 10 μmol/L (\( P = 0.3682 \) and 0.3140, respectively).

**Discussion**

If blood samples are stored under ambient conditions before centrifugation, Hcy production by erythrocytes leads to an increase in plasma concentrations (16, 17). Previously, 10% per hour has been quoted as the initial rate of Hcy production after venipuncture; a similar average rate was observed in the present study (8% per hour). However, an inverse relationship existed between baseline Hcy and the rate of Hcy production (expressed as a percentage of the initial Hcy). Consequently, because a 5 μmol/L increase in Hcy may be associated with a 80% increased risk of vascular disease (9), strict sampling conditions must be observed to prevent false increases in plasma concentrations.

Storage on ice before centrifugation may stabilize plasma Hcy for up to 6 h (20), although current recommendations suggest that processing should occur within 1 h. As we were interested in providing a solution to sample collection away from a centralized laboratory, we chose to look at refrigerated conditions. However, for samples in which EDTA alone was used, the maximum stability was only 3 h.

If ice is unavailable, acid citrate may also provide...
stability for up to 6 h at “room temperature”. However, at higher temperatures, samples are less stable (24). Ducros et al. (18) found that sample stability also depends on the method of analysis. Chromatographic methods showed sample stability for at least 4 h in acid citrate at room temperature, whereas Hcy results increased over the same time course with an immunochemical method (fluorescence polarization immunoassay). Similar findings were reported by Salazar et al. (23) and O’Broin et al. (22), who showed a significant increase after 2 h or a 10% increase after 6 h, respectively. Whereas chromatographic methods measure Hcy itself or a Hcy derivative, immunochemical methods are indirect. Hcy is estimated by the amount of its metabolic precursor, S-adenosylhomocysteine, formed during the assay by the reverse reaction of the enzyme SAHH. In acid citrate, the low pH may prevent Hcy build up by inhibiting SAHH, but precursor build up is not prevented, thereby giving the impression of sample instability. Where stability is reported, there are conflicting reports about whether acid citrate increases (22, 24) or decreases (23) baseline Hcy compared with EDTA samples kept on ice. This confusion may be related to corrections for dilution because acid citrate is added as a liquid, requiring hematocrit estimates. Either way, separate reference intervals are required for interpretation.

Similar problems complicate Hcy measurements on capillary whole-blood lysates (27–29). The method appears quite attractive: a simple fingerprick followed by cell lysis, with stability for 2 days at ambient temperature provided by deactivation of the enzymes that produce Hcy. However, even after correction for dilution caused by the lysing agents, results are lower than in plasma because of further dilution by low intracellular Hcy concentrations.

Sodium fluoride samples at 2–3 h after collection have shown Hcy concentrations similar to baseline EDTA values (25, 26). On closer inspection, however, Hcy continued to increase over time. The effect was attributable to an initial concentration drop caused by the formation of hypertonic saline, which led to fluid shifts. This observation was confirmed by Hughes et al. (30), who saw a sodium fluoride concentration-dependent decrease in hematocrit. Fluoride inhibits anaerobic glycolysis and, therefore, ATP production, which is required for methionine conversion to S-adenosylmethionine, the first step in Hcy production. However, Hcy production may continue because of cellular reserves of S-adenosylmethionine, first postulated by Andersson et al. (16).

Despite these studies, each method has its own weaknesses. After the publication by al Khafaji et al. (31), we investigated the production of evacuated blood tubes containing 3DA. These tubes promised stability for up to 72 h under ambient conditions, without recalculation of Hcy results or changes to currently accepted reference

### Table 1. Percentage and absolute changes in plasma Hcy concentrations in EDTA whole blood over 72 h of storage at 20–25 °C.

<table>
<thead>
<tr>
<th>Hcy values, μmol/L</th>
<th>Initial Hcy, μmol/L</th>
<th>Absolute change, μmol/L</th>
<th>Percentage change</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to &lt;5</td>
<td>4.5 (0.1)</td>
<td>23.8 (1.6)</td>
<td>526.9 (36.7)</td>
<td>9</td>
</tr>
<tr>
<td>5 to &lt;10</td>
<td>6.8 (0.2)</td>
<td>26.1 (1.2)</td>
<td>397.0 (22.0)</td>
<td>34</td>
</tr>
<tr>
<td>10 to &lt;15</td>
<td>12.7 (0.6)</td>
<td>29.8 (4.0)</td>
<td>241.9 (37.2)</td>
<td>7</td>
</tr>
<tr>
<td>15 to &lt;20</td>
<td>17.1 (0.8)</td>
<td>20.8 (3.6)</td>
<td>121.2 (20.6)</td>
<td>4</td>
</tr>
<tr>
<td>20 to &lt;25</td>
<td>23.0 (0.9)</td>
<td>20.0 (0.9)</td>
<td>87.5 (5.7)</td>
<td>3</td>
</tr>
<tr>
<td>25 to &lt;30</td>
<td>27.5 (1.4)</td>
<td>19.8 (2.7)</td>
<td>72.7 (13.6)</td>
<td>2</td>
</tr>
</tbody>
</table>

* Results are mean (SE).

### Table 2. Plasma Hcy concentrations in whole blood over time, subdivided into initial values of ≤10 μmol/L (n = 43) and >10 μmol/L (n = 16), for each storage condition.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Storage temperature, °C</th>
<th>Initial Hcy, μmol/L</th>
<th>Mean (SE) Hcy concentration, μmol/L, at incubation time of</th>
<th>P *</th>
<th>ω</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
<td>3 h</td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>EDTA</td>
<td>20–25</td>
<td>≤10</td>
<td>6.3 (0.2)</td>
<td>8.0 (0.3)b</td>
<td>10.0 (0.3)b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;10</td>
<td>17.6 (1.4)</td>
<td>19.4 (1.5)</td>
<td>21.1 (1.3)</td>
</tr>
<tr>
<td>DS30 Hcy blood tubes</td>
<td>20–25</td>
<td>≤10</td>
<td>6.2 (0.3)</td>
<td>6.2 (0.3)</td>
<td>6.2 (0.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;10</td>
<td>17.3 (1.5)</td>
<td>17.1 (1.4)</td>
<td>17.3 (1.5)</td>
</tr>
<tr>
<td>EDTA</td>
<td>2–8</td>
<td>≤10</td>
<td>6.4 (0.2)</td>
<td>6.6 (0.3)</td>
<td>6.9 (0.3)b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;10</td>
<td>17.5 (1.5)</td>
<td>18.1 (1.5)</td>
<td>18.7 (1.4)b</td>
</tr>
<tr>
<td>DS30 Hcy blood tubes</td>
<td>2–8</td>
<td>≤10</td>
<td>6.2 (0.2)</td>
<td>6.1 (0.2)</td>
<td>6.1 (0.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;10</td>
<td>17.2 (1.5)</td>
<td>17.4 (1.4)</td>
<td>17.0 (1.5)</td>
</tr>
</tbody>
</table>

* P values calculated using one-way within-subject ANOVA (repeated measures).

b Significant differences from 0 h by Tukey’s ω-procedure (α = 0.05).

c Highly significant.

d Not significant.
ranges. However, as observed with acid citrate, stability was temperature-dependent (32). In pilot studies at 20–25 °C, plasma Hcy increased from a mean of 8.5 μmol/L to 11.5 μmol/L over 72 h, an increase of 35%, which conflicted with the 10% increase previously reported (31). Even with a 3DA concentration of 200 μmol/L, Hcy production could not be prevented (D.M. Hill and A.C. Kenney, unpublished results). We therefore considered a combination of SAHH inhibition by use of 3DA and a slowing of Hcy precursor production by chilling to 2–8 °C. Controls were used to ensure that pilot study results were confirmed and to verify that chilling alone was not sufficient for sample stabilization.

Because volunteers were recruited to observe the effects of Hcy stabilization at a range of baseline Hcy values, ANOVA was performed within subjects. Only samples that were collected in DS30 Hcy blood collection tubes and stored refrigerated (2–8 °C) showed stability over 72 h.

The mean plasma Hcy for each storage condition over time is shown in Fig. 1. Under ambient conditions (20–25 °C), the rapid increase in Hcy in samples collected into EDTA alone is clearly visible. Even at 72 h, Hcy production is evident. Therefore, if whole blood is left at room temperature for only a few hours without a preservative to stabilize Hcy, false-positive results may be reported. Chilling samples or storage at ambient temperature in the presence of a SAHH inhibitor (3DA) may both stabilize Hcy to some degree. In fact, 3DA at 20–25 °C stabilizes samples for 6 or even 24 h, depending on the initial Hcy concentration. This offers advantages for samples collected near the site of processing, providing some relief in the requirement to have ice on hand and to deliver the sample quickly to the laboratory. However, if there is a longer delay in transport, refrigerated storage in DS30 Hcy blood collection tubes can offer prolonged stability for 72 h and possibly up to 1 week.

Sample cooling may slow the processes involved in Hcy production. This theory appears to be supported by the data shown in Fig. 1, where a slow but steady increase in plasma Hcy occurs over 72 h in EDTA samples stored at 2–8 °C. In contrast, 3DA inhibits Hcy production in the early period under ambient conditions. However, the block is at the final stage in Hcy production. As the precursors to Hcy accumulate, SAHH inhibition by 3DA is finally overcome. A combination approach appears to be more effective, as chilling will prevent the build up of S-adenosylhomocysteine.

Despite the wide range of initial Hcy values observed, the absolute increase in Hcy in unpreserved samples showed very little difference after 72 h of storage. Consequently, we observed significant differences in the percentage change over time that showed an inverse relationship to baseline Hcy. Similar phenomena have been noted previously over 24 h by Fiskerstrand et al. (19) and most recently over 4 h by Duarte et al. (37). It follows that the thiol pool or the rate of Hcy production must be very similar within the collected samples. Andersson et al. (16) suggested that Hcy in whole-blood samples may be produced from a preformed pool of S-adenosylmethionine. If this is the case, it appears that the concentration of this pool is very similar within blood samples, is independent of Hcy concentration, and therefore, is independent of the efficiency of remethylation in vivo.

We observed significant differences in the degree of error in measurements when samples were stored for prolonged periods without a preservative, according to baseline Hcy concentrations (Table 1). Investigators and clinicians must be aware of this effect when claims are made about stability.

This trial was designed to investigate stability in as wide a range of starting Hcy concentrations as was possible to obtain from an unscreened group, such that stability could be compared in a healthy population vs a group that could be defined as having increased Hcy. When the sample population was divided into two groups, those with higher initial Hcy concentrations were stable over longer periods, e.g., up to 24 h compared with up to 6 h, in the Drew DS30 Hcy collection tubes stored at 20–25 °C. Moreover, any concern over whether significant differences in Hcy had been masked in the samples that were stored at 2–8 °C in DS30 Hcy blood tubes because of the presence of individuals with high baseline Hcy concentrations was unfounded.

We report the first commercially available blood collection tube to allow stability of plasma Hcy in whole blood over the course of 3 days without the need for centrifugation. Although we realize that the requirement for refrigeration is not ideal, several samples may be collected and stored in doctors’ offices or in mobile clinical trial facilities before they have to be transported to a centralized laboratory for processing, thereby facilitating wider population screening. If refrigerated conditions are not readily available, the use of DS30 Hcy blood collection tubes may offer sample stability over 6 h, within a hospital environment, which may be particularly useful in cardiovascular clinics. These tubes can be used with a range of methods, providing that the method does not rely on the action of SAHH; consequently, tubes containing 3DA should not be used in some of the immunochemical methods currently available (38, 39).

Ultimately, our aim is to produce a method of whole-blood collection that allows transport at a range of ambient temperatures. In the interim, we have developed a method that allows samples to be collected and stored for up to 72 h before laboratory intervention. We have successfully used this procedure to offer a mail-in Hcy test where local testing was not available.

We thank all those who helped in the course of this study, including Dr. Paul Haggart, who assisted in collection of blood samples at Birmingham Heartlands Hospital; F. Giltrap and R. Hadley, who assisted in sample handling.
and analysis; and R.E. Ashby (Cranfield University, UK), who helped with the statistical interpretation of the data collected. This study was funded entirely by Drew Scientific Ltd.

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