Short-term and long-term variability of plasma homocysteine measurement

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Within-person and methodological variability of a given analyte are important elements in determining whether an individual has altered concentrations of that analyte. We report the short-term (1 month) within-person, between-person, and methodological variability of plasma homocysteine in 20 healthy participants from whom samples were drawn weekly for 4 weeks. The short-term between-person variance was high, whereas within-person and methodological variances were relatively very low, giving a high reliability coefficient (R) for homocysteine (R = 0.94). The long-term (30 months) reliability coefficient was 0.65, but was greatly influenced by an outlier (R = 0.82 with the outlier excluded). The data suggest that an individual’s plasma homocysteine concentration is relatively constant over at least 1 month, and a single measurement characterizes the average concentration reasonably well.

INDEXING TERMS: hyperhomocysteinemia • cardiovascular diseases • variation, source of • reliability coefficient

Hyperhomocysteinemia, which can result from an inherited deficiency of cystathionine β-synthase or methyltetrahydrofolate reductase activity or from a deficiency of cofactors for these enzymes, is rapidly being recognized as a risk factor for premature vascular diseases—peripheral, cerebral, and coronary [1-6]. Several studies have now shown that subjects who develop these diseases have higher than usual plasma concentrations of homocysteine. Fortunately, homocysteine concentrations can often be lowered, either by dietary manipulations or by supplementation with vitamins (B12, folate, and pyridoxine), thus hypothetically reducing the risk of premature vascular disease [5].

To correctly classify an individual on the basis of laboratory measurement of any disease-related risk factor or to monitor therapy by measurement of that risk factor, in this case homocysteine, knowledge of within-person and methodological variability is extremely important. Analytes with large within-person and methodological variability almost always show weak associations with any given disease. Although between-person variability data for homocysteine have been reported in the literature [7, 8], data for within-person variability are not available. In the present study, we report within-person, between-person, and methodological variances for plasma homocysteine.

Materials and Methods

Subjects
Participants (healthy volunteers working for the University of Minnesota) were 6 men and 14 women, whose mean age was 34.7 years (range 21–65; 35.5 for men and 34.4 for women); 2 men and 4 women were older than 45 years. A standardized protocol (approved by our Institutional Human Subjects Committee) was used to collect blood into EDTA-containing tubes, one tube of blood from each fasting participant. We mixed the tubes’ contents immediately after phlebotomy and kept them on ice until separating the plasma by centrifugation at 4°C and 3000g for 15 min. All of the samples were processed within 1 h of collection. Plasma from each participant was divided into two aliquots, both of which were frozen at −70°C until analyzed for homocysteine. To study short-term, within-person variation, we performed the same protocol on four occasions 1 week apart over ~1 month. For long-term, within-person variation, we collected blood after 30 months from nine of the volunteers who were still available: four men and five women, whose mean age at baseline had been 43 years.
EXPERIMENTAL DESIGN
For estimation of short-term sources of variability, the 160 aliquots from week 1 through week 4 were randomized and divided into 4 batches of 40. Different batches were run at least 1 week apart. For long-term, within-person variability, duplicate samples from 9 participants were run along with their respective samples from week 2. To verify methodological analytical stability, the mean difference between samples from week 2 measured at two occasions was calculated and was 0.24 μmol/L (95% confidence interval: −0.33, 0.79).

ANALYSIS OF HOMOCYSTEINE
Total homocysteine was measured by the method of Ubbink et al. [9], with minor modifications. To 150 μL of plasma we added 10 μL of internal standard [0.1 mmol/L N-(2-mercaptopropionyl)-glycine] and 20 μL of reducing agent (tri-n-butylphosphine, 100 mL/L in dimethylformamide) and incubated the mixture at 4 °C for 30 min. Proteins were precipitated by addition of 150 μL of cold trichloroacetic acid (100 g/L) in 1 mL/L EDTA solution, and the protein-free supernatant was obtained by centrifugation at −10 000g for 5 min. To derivatize the thiols (including homocysteine) in 100 μL of clear supernatant, we added 100 μL of a thiol-specific reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, 1.0 g/L in borate buffer (20 μL of 1.55 mol/L sodium hydroxide and 250 μL of 0.125 mol/L borate buffer in 4 mmol/L EDTA, pH 9.5), and heated this for 1 h at 60 °C.

The chromatographic system consisted of a 15 x 4.6 mm Supelco (Bellefonte, PA) LC-18-DB reversed-phase column, a Varian (Palo Alto, CA) 5000 pump, a Waters (Milford, MA) WISP 710B autoinjector, and GTI/SpectroVision FD-100 fluorescence detector (GTI, Concord, MA). The mobile phase consisted of 0.1 mol/L phosphate buffer (pH 2.05) and acetonitrile (95:5, by vol), and the mobile phase consisted of 0.1 mol/L phosphate buffer (pH 9.5), and heated this for 1 h at 60 °C.

We also computed a closely related measure that is more widely used in epidemiology literature, the ratio of between-person variance to total observed population variance and was also computed from the above variance components, as follows:

\[
R = \left( \frac{\sigma^2_{BP}}{\sigma^2_{Total}} \right) = \frac{\sigma^2_{BP}}{\left(\sigma^2_{BP} + \sigma^2_{WP} + \sigma^2_{M}\right)}
\]

For within-person variability, we examined the homogeneity of the SDs for all 8 measurements of samples from the individual participants (4 weekly samples x 2 aliquots) [12]. The median and the 90th percentile values of the within-person CVs (CV_{p90} and CV_{p50}, respectively), and their corresponding critical differences (d_{50} and d_{90}) were then calculated according to the methods described by Costongs et al. [13].

We also computed a closely related measure that is more widely used in the clinical chemistry literature, the index of individuality, as SD(WP + M)/SD_{BP}, where SD_{BP} is (\sigma^2_{BP})^{1/2} and SD(WP + M) is (\sigma^2_{WP} + \sigma^2_{M})^{1/2}.

The minimum real change detectable with 95% confidence in two sequential homocysteine measurements for a single patient was also calculated [14] from within
person and methodological variances, as $2.77(\sigma^2_{\text{WP}} + \sigma^2_{\text{M}})^{1/2}$.

Because there was no systematic change in plasma homocysteine values in nine participants examined after 30 months, we also used the nested random effects analysis of variance model to estimate the long-term reliability of measurement.

**Results**

As shown in Table 1, plasma homocysteine concentrations ranged from 3.71 to 17.53 μmol/L in the 20 subjects, with a mean of 8.42 μmol/L. The mean plasma homocysteine concentration in men was significantly higher than that in women (9.92 vs 7.79 μmol/L; $P < 0.001$). In men, the mean plasma homocysteine was 9.71 μmol/L for ages ≥ 45, 10.02 for ages ≤ 45 ($P = 0.7$). In women of those ages, the means were 9.65 and 7.04 μmol/L, respectively ($P < 0.001$). The Pearson correlation coefficient between age and homocysteine concentration was 0.24 ($P < 0.01$). Linear regression showed that both age and sex were significantly associated with plasma homocysteine concentrations.

Between-person variance was relatively high, whereas within-person and methodological variances were relatively very low. The short-term reliability coefficient ($R$) of homocysteine therefore was excellent, with $R = 0.94$ for the entire group of participants. It remained high, even when the homocysteine concentrations were analyzed by age ($R = 0.96$ and 0.85 for men and 0.90 and 0.89 for women, for ages ≤ 45 and > 45, respectively) and sex (0.96 for men and 0.92 for women). The high short-term repeatability was also reflected in the CV, the index of individuality, and the minimum detectable difference.

In the long-term variability study (Fig. 1), we determined that the mean difference in plasma homocysteine concentration for 9 participants at 30 months vs week 2 was $-0.03 \mu mol/L$ (95% confidence interval: $-1.25, 1.19$); excluding one outlier, it was 0.52 ($-0.13, 1.17$). The reliability coefficient remained high ($R = 0.65$ for all 9 participants, 0.82 after excluding the outlier).

Because the within-person SDs were not homogeneous, we also calculated the critical differences at $CV_{p50}$ and $CV_{p90}$. $d_{k50}$ and $d_{k90}$ were 24.6% and 32.0%, respectively (Table 1).

**Discussion**

In epidemiological and clinical studies, between-person differences are used to relate various measured risk factors to disease occurrence. Because within-person and methodological variability simply add unwanted noise, it is desirable that these variations be small. Nevertheless, variability does exist and can affect the interpretation of the relation between a particular risk factor and disease occurrence, therefore making it important to quantify the magnitude of these variance components.

The reliability coefficient, the ratio of between-person variance to the total observed population variance, is numerically equivalent to the correlation coefficient of repeated measurements made on blood collected and

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**Table 1. Short-term variability components for homocysteine measurement.**

<table>
<thead>
<tr>
<th>Homocysteine, μmol/L</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall mean</td>
<td>8.42</td>
</tr>
<tr>
<td>SD</td>
<td>2.84</td>
</tr>
<tr>
<td>Range of means for the 20 subjects</td>
<td>3.71–17.53</td>
</tr>
</tbody>
</table>

**Variance components, μmol/L (df = 19)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>8.43</td>
</tr>
<tr>
<td>Between-person</td>
<td>7.94</td>
</tr>
<tr>
<td>Within-person</td>
<td>0.35</td>
</tr>
<tr>
<td>Method</td>
<td>0.13</td>
</tr>
<tr>
<td>Reliability coefficient, $R$</td>
<td>0.94</td>
</tr>
<tr>
<td>CV, %</td>
<td></td>
</tr>
<tr>
<td>Between-person</td>
<td>33.5</td>
</tr>
<tr>
<td>Within-person</td>
<td>7.03</td>
</tr>
<tr>
<td>Method</td>
<td>4.30</td>
</tr>
<tr>
<td>Within-person + method</td>
<td>8.25</td>
</tr>
</tbody>
</table>

**Distribution of within-person CVs, %**

| CVp50, %                  | 7.56  |
| 90th percentile (CVp90)   | 10.48 |

**Critical difference, $d_{k}$, %**

| Based on CVp50 ($d_{k50}$) | 24.6  |
| Based on CVp90 ($d_{k90}$) | 32.0  |

**Index of individuality**

| Minimum detectable difference in two measurements at 95% confidence, μmol/L | 1.93 |

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*aBased on two split aliquots from four weekly blood samplings in 20 subjects.

*bAlthough the design allowed for separation of between-batch and within-batch methodological variances, these were small and therefore were pooled.

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**Fig. 1.** Correlation of plasma homocysteine concentrations (μmol/L) in nine participants at baseline vs 30 months later. The data from replicate measurements were pooled to get the mean concentrations.
analyzed in a laboratory at multiple time points; it can vary from 0 to 1. Values approaching unity are desired. For an analyte with very high reliability coefficient, a single measurement of the analyte in a study participant will correctly classify that participant with respect to his or her short-term average analyte concentration, which Fraser and Harris have termed an individual’s “homeostatic set point” [15]. On the other hand, if the reliability coefficient is low, a single measurement of the analyte may not predict the real concentration or change in analyte concentration and thus may not predict disease occurrence. Previous studies from our laboratory and others have shown that reliability coefficients for commonly used chemistry analytes fall between 0.6 and 0.9 [16].

In this study, the short-term reliability coefficient for homocysteine was high (R = 0.94) relative to many more common chemistry analytes [16], suggesting that, in the short-term, single homocysteine measurements may classify persons with respect to their average plasma homocysteine concentrations quite well. In other words, a typical individual’s homocysteine results appear to stay within a relatively narrow range for at least 1 month. This situation is also delineated by the low value of index of individuality (0.25). As might be expected, the long-term (30 months) reliability coefficient for homocysteine was lower (R = 0.65), although this finding was based on results for only nine subjects. This result may be due to long-term variations in diet, particularly the intake of certain vitamins (pyridoxine, folate, and vitamin B₁₂) that are obligatory cofactors of enzymes in the homocysteine metabolic pathways [2, 5, 17]. Just as Andersson et al. had reported earlier [7], we also found higher concentrations of homocysteine in men than in women, as well as higher concentrations in the subjects older than 45 years vs those younger than 45.

Several methods for homocysteine measurement are available [5, 18-21], and our methodological variances are comparable with the variances reported by others. Homocysteine measurement, like many analytes measured in clinical laboratories, does not quite meet the widely quoted goal for analytical precision (i.e., methodological total SDs should be <0.5 within-person SD: CV_M ≤0.5 CV_WP); nonetheless, we consider its precision to be reasonably acceptable clinically. Efforts for improving homocysteine measurements and reducing methodological SDs should continue to make a single measurement of homocysteine more reliable.

In conclusion, the high reliability coefficient shows that homocysteine concentrations in an individual are relatively constant, such that a single measurement well characterizes an individual’s average concentration of homocysteine over at least 1 month. Even the long-term reliability (over a 30-month period) is relatively high. This work was in part supported by a Van Slyke Society Research Grant from the American Association for Clinical Chemistry.

References
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