Background and Objectives. The aim of this study was to compare the performance of a commercially available IMx immunoassay with that of a reversed-phase high performance liquid chromatography (HPLC) method for measuring plasma total homocysteine (tHcy).

Methods. The levels of tHcy before and after oral methionine loading (ML) were measured in 135 healthy subjects and 39 patients scheduled for routine tHcy determination. The IMx method uses fluorescence polarization immunoassay (FPIA) technology. The HPLC-method includes derivatization with ABD-F and post-column fluorescence detection.

Results. The imprecision was very low with both methods for both normal (11 µmol/L) and high (29 µmol/L) tHcy levels. The within and between-run coefficients of variation were < 5%. Both methods were able to discriminate between similar concentrations of tHcy both at normal and moderately high levels. There was a good correlation between measurements obtained with the two methods (r = 0.985, p = 0.001). The mean levels of tHcy measured with the IMx assay tended to be slightly higher than those with the HPLC both in the fasting state (mean difference = 0.8 µmol/L) and after ML (5.3 µmol/L). However only the difference in post-ML levels was statistically significant (p < 0.001). The percentage of patients with hyperhomocysteinemia identified with the two methods was similar.

Interpretation and Conclusions. The IMx method compares well with an established HPLC method for measurement of fasting tHcy plasma levels. ©2002, Ferrata Storti Foundation

Key words: homocysteine, methods, thrombosis.


oratories (Abbott Park, IL, USA). L-homocystine, L-cystine, tri-n-butylphosphine (TBP), and 7-fluoro-2,1,3-benz-oxadiazole-4-sulfonamide (ABD-F) were from Sigma (St. Louis, MO, USA).

Subjects

Plasma samples from 39 patients with previous episodes of venous or arterial thrombosis (21 men, 18 women, median age 51 years) and 135 healthy subjects (61 men, 74 women, median age 45 years) were analyzed by both the IMx Hcy assay and the HPLC method.

Blood samples

Venous blood samples for tHcy measurement were taken from each subject before and 4 hours after oral methionine loading (3.8 g/m² b.s.a.). They were drawn into vacuum tubes containing K3-EDTA, immediately placed on ice and centrifuged at 2,200 × g at 4°C for 20 minutes within 1 hour; the supernatant platelet-poor plasma was stored at -80°C until assay.

Assay methods

The IMx Hcy assay is based on reduction of the plasma samples with dithiothreitol (DDT) and subsequent conversion of free Hcy to S-adenosyl homocysteine (SAH) by SAH hydrolase in the presence of added adenosine. The sample and the tracer (fluoresceinated SAH analog) compete for binding to monoclonal anti-SAH antibody. This reaction is followed by detection of SAH by a fluorescence polarization immunoassay; the concentration of tHcy in plasma is inversely related to the intensity of the polarized light.3 The HPLC method4 includes reduction of the plasma samples with tri-n-butylphosphine, pre-column derivatization with ABD-F and fluorescence detection after separation by reversed-phase HPLC.

Parameters studied

Imprecision. Two pooled plasma were prepared by mixing EDTA-plasma from healthy subjects. The two pools were prepared from plasma collected before (A) and 4 hours after oral methionine loading (P) (3.8 g/m² b.s.a.). The concentration of tHcy was 11 µmol/L in pool A and 29 µmol/L in pool P as measured by the HPLC method. The within-run coefficient of variation (CV) for the two methods was obtained by assaying the two pools (A and P) 18 times in the same run. The between-run CV was obtained by assaying the two pools in each run over 18 days.

Recovery. Three plasma samples containing moderately high tHcy levels (38.4, 36.2, 35.9 µmol/L), as determined by HPLC, were diluted with the phosphate buffer from 0 to 32-fold. Linear regression of the measured tHcy (x) versus the expected tHcy (y) could be described by the following equation: y = 1.01 x - 0.982 (r² = 0.998) (Figure 1).

Discrimination. Both methods identified pool A as the pool with the highest (normal) tHcy concentration and pool P as the pool with the highest (moderately high) concentration. The discrimination between the two

Results

Imprecision

The imprecision was very low for both methods: the within-run CV for fasting and post-ML values were 2.1% and 1.2% with the HPLC method, and 1.9% and 1.4% with the Abbott IMx Hcy assay; the between-run CV were 4.6% and 2.7% with HPLC and 2.5% and 2.2% with the IMx Hcy assay.

Recovery

Three plasma samples containing moderately high tHcy values (38.4, 36.2, 35.9 µmol/L), as determined by HPLC, were diluted with the phosphate buffer from 0 to 32-fold. Linear regression of the measured tHcy (x) versus the expected tHcy (y) could be described by the following equation: y = 1.01 x - 0.982 (r² = 0.998) (Figure 1).

Discrimination

Both methods identified pool A as the pool with the highest (normal) tHcy concentration and pool P as the pool with the highest (moderately high) concentration. The discrimination between the two
normal and moderately high) concentrations was statistically significant with both methods (Table 1).

Method comparison

The correlation between tHcy levels measured in 270 samples from 135 healthy subjects (fasting and post-ML levels) with the two methods was good ($r^2 = 0.969$, $p = 0.001$) (Figure 2).

Plots of the differences in tHcy measurements obtained with the two methods as a function of their mean value are shown in Figure 3A. The differences in tHcy levels detected by the two methods increased significantly with increasing mean tHcy levels ($y = -0.25x + 1.69$, $r = -0.826$, $p < 0.0001$). After logarithmic transformation of the data (Figure 3B), the mean differences of tHcy levels detected by the two methods was -0.05 (SD = 0.05), with limits of agreement comprised between -30% and +12%. The concentrations of fasting tHcy measured with the two methods did not differ significantly, whereas the concentrations after methionine loading were significantly higher when measured with the Abbott IMx Hcy assay (Table 2).

The percentage of patients with high fasting levels of tHcy was 38% with the HPLC method and 35% with the IMx Hcy assay ($p = 0.25$) and that with high post-ML tHcy increments above fasting levels was 69% with HPLC and 58% with IMx ($p = 0.02$).

<table>
<thead>
<tr>
<th>Sample</th>
<th>tHcy (µmol/L)</th>
<th>Abbott IMx (n=18)</th>
<th>HPLC (n=15)</th>
<th>$p$ *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool A1</td>
<td>12.5±0.3</td>
<td>11.3±0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool A2</td>
<td>15.7±0.4</td>
<td>13.8±0.6</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Pool P1</td>
<td>31.7±0.5</td>
<td>26.6±0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool P2</td>
<td>34.7±0.8</td>
<td>29.4±1.1</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Discrimination between plasma samples with similar tHcy concentrations.

Means±SD; * t test for paired data (A1 vs A2 and P1 vs P2). See text for details.

<table>
<thead>
<tr>
<th>Table 2. Mean tHcy concentrations measured in plasma from healthy individuals before and 4 hours after an oral methionine load (3.8 g/m² b.s.a.). Comparison between the Abbott IMx Hcy assay and an HPLC method.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurement</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>Fasting levels</td>
</tr>
<tr>
<td>4 h post-ML</td>
</tr>
</tbody>
</table>

Means±SD; *t test for paired samples was used to compare the tHcy levels measured with the two methods.
Discussion

Case-control and cross-sectional studies clearly indicate that mild-to-moderate hyperhomocysteinemia is associated with a heightened risk of thromboembolic diseases. Two important features distinguish hyperhomocysteinemia from other known risk factors for thrombosis: 1) it is associated with increased risk for both arterial and venous thrombosis, and, 2) it can be easily corrected with safe and relatively inexpensive therapeutic approaches, such as dietary supplementation of folic acid and other vitamins (B6 and B12). These features may have a tremendous impact on the epidemiology and natural history of thrombotic diseases and lead many laboratories to include tHcy measurement in the screening procedure to assess the thrombotic risk. In the past, plasma tHcy has been measured by gas chromatography-mass spectroscopy, or HPLC with fluorimetric or electrochemical detection. Though reliable, these methods require experience and equipment that are not easily available in general clinical laboratories. More recently, a fully automated immunoassay for measurement of tHcy based on fluorescence polarization immunoassay detection with the Abbott IMx analyzer has been introduced. Therefore, we evaluated the FPIA immunoassay and compared the results obtained with this immunoassay with those of a home-made HPLC method.

With the IMx Hcy assay, there was a very good linearity of recovery for values comprised between 2 to 40 µmol/L. The reproducibility of the IMx Hcy assay proved to be excellent both at normal and moderately high tHcy concentrations, in accordance with previous reports in the literature. This may eliminate the need to make duplicate determinations.

Another performance characteristic that was investigated in this study was the ability of the two methods to discriminate between similar concentrations of tHcy in the test sample. This is important because the association between tHcy concentration and the cardiovascular risk is graded, with a calculated increase in risk of 40 % for every 5 µmol/L increase in tHcy concentration. Both methods were able to discriminate between two close tHcy concentrations, both at normal and moderately high levels.

Our study confirms the results of previous reports, showing that both the fasting and the post-ML concentrations of tHcy measured by the IMx Hcy assay tended to be slightly higher than those measured with the HPLC method, although only the difference in the post-ML concentrations was statistically significant. We identified four possible reasons for such differences:

Figure 3. Differences between results of fasting (open circles) and post-methionine loading (crosses) tHcy measurements with the HPLC and Abbott IMx methods. The mean difference (±2SD) was 3.048 (±6.78) mmol/L on a linear scale (panel A) and 0.05 (±0.1) on a log-transformed scale (panel B).
matrix effects: this hypothesis is, however, unlikely, because the discrepancy was seen also when we measured the tHcy levels in two solutions containing low and moderately high concentrations of L-homocysteine dissolved in 0.01 N HCl (not shown);

2) different analytical principles of the two assays: theoretically, the presence of endogenous S-adenosylhomocysteine (SAH) or S-adenosylmethionine (SAM), which have been reported to increase after oral methionine loading,\textsuperscript{17,18} could contribute to the higher tHcy concentrations measured with the IMx Hcy assay. However, the presence of very low, nanomolar concentrations of these compounds in plasma\textsuperscript{3} makes this hypothesis very unlikely;

3) the photo-sensitivity of ABD-F could lead to underestimation of tHcy concentrations with the HPLC method: however, the analytical CV of the HPLC method compares well with that of the IM x Hcy assay, indicating that the possible effect of photo-sensitivity destruction on the quality of the HPLC results is probably only of minor importance in explaining the lower concentrations of tHcy measured by the HPLC vs IM x Hcy assay;

4) use of different calibrators:\textsuperscript{19} we believe that this might be the most likely explanation for the observed discrepancy between the two methods.

It is important to note that, in spite of the slight differences in tHcy measurements, the concordance of the two methods in diagnosing fasting hyperhomocystinemia in patients with previous thrombotic events was very good, while a significantly lower prevalence of post-ML hyperhomocystinemia was found using the IM x Hcy assay.

In conclusion, the Abbott IMx immunoassay for tHcy measurement compares well with an established HPLC-method and can be used for routine measurement of fasting tHcy plasma levels. The comparability of the two methods is less satisfactory for measurement of the post-ML tHcy levels. Easy operation and complete automation make the Abbott IMx immunoassay a suitable candidate to replace HPLC based methods especially in general clinical laboratories that measure fasting tHcy levels.

Contributions and Acknowledgments

MLZ: drafting the article and revising it critically for important intellectual content; VC: analysis and interpretation of data; AT: conception and design; PMM: final approval of the version to be submitted;

MC: revising the article critically for important intellectual content and final approval of the version to be submitted.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

References


**What is already known on this topic**
Hyperhomocysteinemia has been considered as a thrombosis risk factor, leading to an increasing demand for its measurements in the routine clinical laboratory.

**What this study adds**
This study shows that an easy and automated method, the fluorescence polarization immunoassay, compares well with an established and more complex HPLC-determination.

**Manuscript processing**
This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Vicente and the Editors. Manuscript received June 11, 2001; accepted October 6, 2001.

**Potential implications for clinical practice**
These data suggest that fluorescence polarization immunoassay could be a suitable candidate to replace HPLC-based methods in general clinical laboratories that measure fasting total plasma homocysteine levels.

Vicente Vicente Garcia, Deputy Editor