Laboratory Hematology

Determination of total homocysteine in plasma: comparison of the Abbott IMx immunoassay with high performance liquid chromatography

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Research paper

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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.

Key words: homocysteine, methods, thrombosis.

Homocysteine (Hcy) is a sulfhydryl amino acid derived from the metabolic conversion of the essential amino acid, methionine. It exists both in free and protein-bound forms and is oxidized in plasma to the disulfides homocysteine-homocysteine (homocystine) and homocysteine-cysteine (mixed disulfide). Free and protein-bound Hcy and its disulfides are globally referred to as total homocysteine (tHcy). Hcy can be trans-sulfurated to cysteine or remethylated to methionine through pathways that are catalyzed by a series of enzymes and regulated by vitamins as cofactors, or cosubstrates.[1] Impairment of these metabolic pathways because of enzyme and/or vitamin deficiency may result in accumulation of tHcy in plasma. In the last two decades, a growing amount of interest has focused on mild-to-moderate hyperhomocysteinemia as a risk factor for thromboembolic diseases,[2] leading to an increasing demand for tHcy measurements in the routine clinical laboratory. High pressure liquid chromatography (HPLC) has generally been used for determination of tHcy, but it is time-consuming and requires highly skilled technical staff. Recently, a fully automated immunoassay for measurement of tHcy based on a fluorescence polarization immunoassay (FPIA) detection in the Abbott IMx analyzer has been introduced.

The aim of this study was to compare the performance of the IMx Hcy assay to that of an established HPLC-method for plasma tHcy measurement.

Design and Methods

Materials

The IMx analyzer, disposable cuvettes and cartridges, the FPIA buffer, the reagent pack, calibrators and controls were obtained from Abbott Lab-

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/m^2 body surface area-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 method[4] 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 made from plasma collected before (A) and 4 hours after oral methionine loading (P) (3.8 g/m^2 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. This was assessed by measuring tHcy levels in plasma samples with moderately high tHcy levels, after serial dilutions with phosphate buffer. The measured values were compared to expected values.

Discrimination. The ability to discriminate between similar tHcy concentrations was evaluated by testing for significant differences of tHcy levels of paired pooled plasma with similar, but not identical tHcy concentrations. The two paired pooled plasmas were prepared by dividing pool A and P (described above) into two equal portions coded as A_1, A_2, P_1 and P_2. Suitable volumes of pool A_2 and pool P_1 were removed from the stock and exchanged (P_1 in A_2 and A_2 in P_1). As a result, the concentrations of tHcy within each pair of pools (A_1-A_2 and P_1-P_2), obtained by HPLC, were similar but not identical (11.3 - 13.8 µmol/L and 26.6 - 29.4 µmol/L). The four pools (A_1-A_2 and P_1-P_2) were analyzed in each run over 18 days.

Method-comparison. tHcy concentrations for plasma from 135 healthy subjects (fasting and post-ML levels) were measured by the IMx Hcy assay and the HPLC method. Results were compared by linear regression analysis, and by plotting the difference for tHcy measurements (HPLC – IMx) versus average concentration according to Bland and Altman.[5]

Finally, we compared the percentage of patients with hyperhomocysteinemia detected by the two methods. Hyperhomocysteinemia was defined as tHcy levels higher than the 95th centile of distribution of normal values.

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: $y = 1.01\ x - 0.982$ ($r^2 = 0.998$) (Figure 1).

Discrimination

Both methods identified pool A_2 as the pool with the highest (normal) tHcy concentration and pool P_2 as the pool with the highest (moderately high) concentration. The discrimination between the two

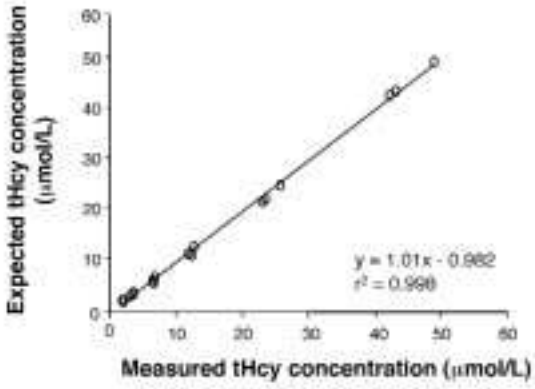

Figure 1. Recovery of tHcy with the Abbott IMx Hcy assay. Plot of the linearity of expected vs measured tHcy concentrations (μmol/L).

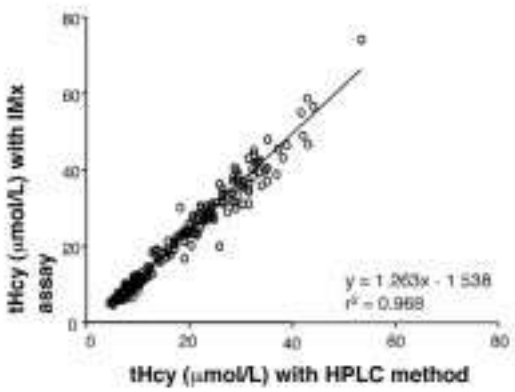

Figure 2. Correlation between the tHcy measurements obtained with a HPLC method (x-axis) and the Abbott IMx method (y-axis) (n = 270).

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

	tHcy (μmol/L)	
Sample	*Abbott IMx (n=18)*	*HPLC (n=15)*
Pool A_1	12.5±0.3	11.3±0.5
Pool A_2	15.7±0.4 p<0.0001	13.8±0.6 p<0.0001
Pool P_1	31.7±0.5	26.6±0.8
Pool P_2	34.7±0.8 p<0.0001	29.4±1.1 p<0.0001

*Means±SD. * t test for paired data (A_1 vs A_2 and P_1 vs P_2). See text for details.*

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.

		tHcy (μmol/L)		
Measurement	*n*	*AbbottIMx*	*HPLC*	*p**
Fasting levels	135	10.2±4.6	9.4±3.6	0.11
4 h post-ML	135	30.7±9	25.4±6.9	<0.0001

*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 hyperhomocystinemia from other known risk factors for thrombosis: 1) it is associated with increased risk for both arterial[6-9] and venous[10] 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).[11] 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 spectrography, or HPLC with fluorimetric or electrochemical detection.[12] 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.[13-15] 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.[7] 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,[14,16] 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:

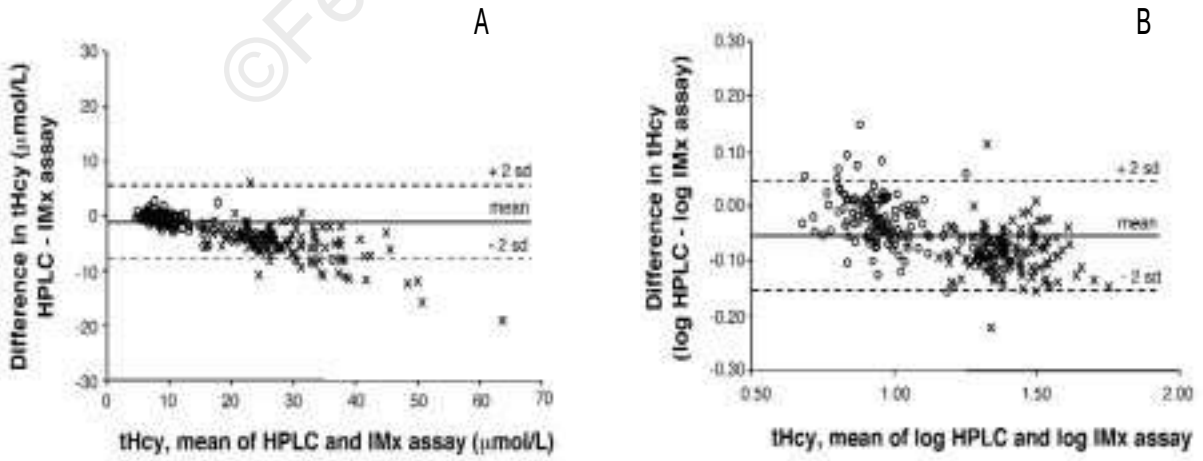

Figure 3. Differences between results of fasting (open circles) and post-methionine loading (crosses) tHcy measurements with the HPLC and Abbott IMx methods. The mean difference (±2SD) was 3.048 (±6.78) mmol/L on a linear scale (panel A) and 0.05 (±0.1) on a log-transformed scale (panel B).

1) *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-homocystine 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,[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[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 IMx 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 IMx Hcy assay;
4) *use of different calibrators*:[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 IMx 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.

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Peer Review Outcomes

What is already known on this topic

Hyperomocysteinemia 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