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Interlaboratory Variation of Plasma Total Homocysteine Measurements: Results of Three Successive Homocysteine Proficiency Testing Surveys

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Background: **Numerous studies have demonstrated that increased plasma total homocysteine (tHcy), whether measured after fasting or after a methionine load, is associated with increased risk for cardiovascular and thromboembolic diseases. However, little information is available regarding interlaboratory variation of tHcy measurements, especially at the increased tHcy concentrations observed after loading.**

Methods: **We conducted three Homocysteine Proficiency Testing Surveys at 6-month intervals. Sets of five plasma pools with endogenous tHcy concentrations ranging from 5 to 48 μmol/L were sent to participants. We received 11, 23, and 17 responses in the first, second, and third surveys, respectively. The following methods were used by participating laboratories: fluorescence polarization immunoassay (FPIA); HPLC with fluorescent detection (HPLC-FD), further subdivided by type of reduction/derivatization; HPLC with electrochemical detection (HPLC-ED); amino acid analyzer with ninhydrin detection; and liquid chromatography–electrospray tandem mass spectrometry (LC-MS/MS).**

Results: **In surveys 1 and 2, no notable differences among the mean tHcy values obtained by the different methods performed were observed. In survey 3, tHcy values obtained by the FPIA method were significantly lower ($P < 0.05$) at increased tHcy concentrations (34 μmol/L) compared with values obtained by HPLC-FD regardless of reduction/derivatization agents used. Our laboratory confirmed the observation that tHcy values obtained by the FPIA method differed from those obtained by HPLC-FD at increased tHcy concentrations by reanalyzing each pool 10 times by FPIA and HPLC-FD using tributylphosphine–ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate ($P < 0.001$ for tHcy >19 μmol/L). The mean among-method variations in surveys 1, 2, and 3 were 19%, 12%, and 9.6%, respectively. When results of the three surveys were combined, the mean among-method variation on 170 samples was 13%. Within-method variation was lowest for the FPIA method (4.4%), and ranged from 11–20% for HPLC methods.**

Conclusions: **Various degrees of imprecision and lack of correlation among tHcy methods indicate that there is a need to improve analytical precision, decrease analytical difference, and standardize tHcy measurements among laboratories.**

In recent years, the measurement of plasma total homocysteine (tHcy)[1] and its association with cardiovascular and thromboembolic diseases have received considerable attention. In the last decade, numerous epidemiologic studies have demonstrated that moderately increased tHcy, whether measured after fasting or 2–6 h after a methionine load, is associated with an increased risk for coronary artery disease (CAD) *(1–5)*. It has also been indicated that plasma tHcy concentrations are a strong predictor of mortality in patients with angiographically

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[1] Nonstandard abbreviations: tHcy, total homocysteine; CAD, coronary artery disease; FD, fluorescent detection; ED, electrochemical detection; FPIA, fluorescence polarization immunoassay; GC-MS, gas chromatography–mass spectrometry; LC-MS/MS, liquid chromatography–electrospray tandem mass spectrometry; PML, post-methionine load; TBP, tributylphosphine; TCEP, tris-2-carboxyethylphosphine; SBDF, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate; and MBrB, monobromobimane.

confirmed CAD *(6, 7)*. In addition, high plasma tHcy concentrations have been reported to be a risk factor for deep-vein thrombosis in the general population *(8–10)*. As a result of these studies, the volume of tHcy testing in clinical laboratories has grown steadily during the last decade.

Initially, tHcy was measured with amino acid analyzers, using the ninhydrin reaction as the method of detection. These methods were generally insensitive, and HPLC with fluorometric detection (HPLC-FD) became the method of choice *(11)*. Such methods are based on the reduction of protein-bound homocysteine, homocystine, and mixed disulfides to reduced homocysteine, followed by derivatization of the reduced homocysteine with thiol-specific fluorogenic reagents. HPLC with electrochemical detection (HPLC-ED) is also used and has the advantage that no derivatization of the sample is required before detection *(11)*. More recently, commercially available automated immunoassays have been introduced. They include the microtiter plate enzyme immunoassay *(12)* and the fluorescence polarization immunoassay (FPIA) on the Abbott IMx® analyzer *(13)*. Both methods are based on enzymatic conversion of homocysteine to *S*-adenosyl-L-homocysteine, which is subsequently detected by a competitive immunoassay. Gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–electrospray tandem mass spectrometry (LC-MS/MS) methods have also recently been reported *(11, 14, 15)*. These methods are used for the routine quantification of tHcy in some laboratories and may be useful in the standardization of plasma tHcy assays. In particular, the LC-MS/MS method has been suggested as a reference method *(11)*.

Despite increased interest in measurement of fasting and post-methionine load (PML) plasma tHcy and the growing number of methodologies used among laboratories, only a few studies on method and interlaboratory variation have been performed. An external quality assessment program including nine laboratories from the Scandinavian countries showed that CVs for the measurement of tHcy by HPLC or GC-MS were 6–12% *(16)*. This program was continued, and a later report including 28 laboratories with 34 sets of results using HPLC, GC-MS, and immunoassays showed an overall within-laboratory CV of 7.5%. Only results obtained with HPLC methods showed significant between-laboratory variance. Nine of the 34 participants achieved the minimum imprecision goal of 6% *(17)*. An international study among 14 laboratories on the comparison of tHcy values obtained on plasma samples with and without added homocysteine showed that mean inter- and intralaboratory variations for HPLC, GC-MS, and immunoassays were <10% *(18)*. In these three studies, increased tHcy was obtained by adding L-homocystine to samples. More recently, a multicenter study on two pairs of pooled plasma of normal fasting and PML tHcy concentrations showed that within-laboratory reproducibility, expressed as median CV, ranged from 2.7% to 3.3% (FPIA), 9.2% to 13.9% (HPLC), and 21.8% to 24.2% (enzyme immunoassay). Between-laboratory variation ranged from 13.9% to 15.6% *(19)*.

To assess the performance, comparability, and within- and among-method variation of tHcy methodologies performed among different laboratories, we invited laboratories to participate in one or more of three Homocysteine Proficiency Testing Surveys. Here we report plasma tHcy results from these surveys, each consisting of sets of five samples with endogenous tHcy concentrations ranging from 5 to 48 μmol/L. Increased concentrations of endogenous tHcy were obtained by methionine loading, thus obviating the need to add L-homocystine to plasma.

Materials and Methods

PARTICIPATING LABORATORIES

We invited laboratories that were involved in tHcy measurement to participate in Homocysteine Proficiency Testing Surveys at 6-month intervals. The surveys were open to all interested laboratories; a minimal fee was paid by each participant to defray costs. The survey was discontinued after 18 months because the College of American Pathologists included tHcy analysis in their surveys.

Participants were asked to measure tHcy in five blinded samples using their own methods and standards. Eleven, 17, and 15 laboratories participated in the first, second, and third surveys, respectively. Eight laboratories provided results from two different methods; thus a total of 51 sets of data were obtained. All but one of the laboratories were within the US. Laboratories were grouped by the type of methodology performed: (*a*) HPLC-FD, further subdivided by the types of reduction [tributylphosphine (TBP), $NaBH_4$, tris-2-carboxyethylphosphine (TCEP)] and derivatization [ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBDF) or monobromobimane (MBrB)] agents used; (*b*) HPLC-ED; (*c*) FPIA; (*d*) amino acid analyzer with ninhydrin detection; or (*e*) LC-MS/MS.

SPECIMENS

Twelve healthy individuals (10 females and 2 males) were recruited for this study. The study was approved by the Human Studies Committee of the University of Minnesota Institutional Review Board, and all individuals gave informed consent. For 5 of the 12 participants, a 200-mL fasting blood sample was drawn into EDTA-containing tubes. The remaining seven individuals were methionine-loaded to obtain high concentrations of endogenous tHcy. For the methionine loading, 100 mL of fasting blood was first collected in EDTA-containing tubes. Methionine (100 mg/kg of body weight) was mixed in 8 ounces (224 mL) of cranberry juice and administered orally, and an additional 100-mL blood sample was collected into EDTA-containing tubes 4 h after loading. Samples were centrifuged at 3000*g* for 10 min at 4 °C within 30 min of collection, and the plasma tHcy concentration was measured by HPLC-FD using TBP/SBDF as the reduction/

derivatization agents, as described previously *(20, 21)*. Plasma aliquots from each participant were mixed to obtain 10 pools with tHcy concentrations ranging from ~5 to 48 μmol/L. Each pool was dispensed as 750-μL aliquots into 2.0-mL Nalge cryovials, randomly coded, and frozen at −70 °C until shipment. Each participating laboratory was sent a shipment on dry ice containing identical sets of five blinded samples randomly selected from the 10 plasma pools; the five sets of samples varied with each survey. Unused aliquots of each pool were stored in our laboratory frozen at −70 °C.

STATISTICAL METHODS

To test for methodologic differences, we grouped participating laboratories by method. Results obtained by individual methods are given as mean values ± SD and CV. Differences between means of results were tested using General Linear Model (GLM) univariate analysis with the SPSS GLM procedure (Release 10.0; SPSS). $P < 0.05$ was considered statistically different.

Results

Shown in Table 1 are results from three surveys conducted at 6-month intervals. In all three surveys, the HPLC method was used most frequently to measure tHcy. In surveys 2 and 3, tHcy was measured by the FPIA method 7 of 23 and 6 of 17 times, respectively.

The means, SDs, and CVs of the tHcy values categorized by method in each of the three surveys are listed in Table 1. When we compared mean tHcy values in survey 1, we found no significant differences in values obtained for the five pools when compared with values obtained with the other methods or the among-method mean. In survey 2, only the mean value of the pool with lowest tHcy concentration (pool A) was significantly lower ($P = 0.027$) by the amino acid analyzer method compared with the mean value obtained by HPLC-FD with TCEP/SBDF as the reduction/derivatization agents. In survey 3, the within-method SD of most methods was smaller than in surveys 1 and 2, and significant differences in mean tHcy values among several HPLC methods at various tHcy concentrations were observed (Table 1). Mean tHcy values obtained by the FPIA method (n = 6) were significantly lower at low tHcy concentrations (7 μmol/L) compared with values obtained by HPLC-FD using $NaBH_4$/MBrB and TBP/SBDF as the reduction/derivatization agents. At increased tHcy concentrations (34 μmol/L), the results obtained by the FPIA method were significantly lower compared with values obtained by HPLC-FD regardless of the reduction/derivatization agents used. When mean tHcy values for each method were compared with the among-method means, only the tHcy values of the HPLC-FD method using $NaBH_4$/MBrB for reduction/derivatization were significantly different for all five pools ($P < 0.01$). tHcy values obtained by LC-MS/MS were extremely close to the among-method mean tHcy values, providing evidence that LC-MS/MS may be an acceptable primary reference method for tHcy measurement.

We compared within- and among-method CVs to assess the interlaboratory imprecision of tHcy measurements. As shown in Table 1, within-method variation ranged from 2.7% to 22%, depending on the method performed. Because of the small number of laboratories included in each method for each of the surveys, the within-method variation data must be interpreted with caution; however, the FPIA method performed in surveys 2 and 3 consistently had lower CVs (mean, 5.9% and 2.7%, respectively) compared with the other methods. The mean CVs among the methods performed in surveys 1, 2, and 3 were 19%, 12%, and 9.6%, respectively.

Five of the 10 pools were analyzed in two of the three surveys; therefore, these results were combined to compare mean tHcy values obtained by different methods and to assess among- and within-method variation on a larger number of samples over a longer period of time. Table 2 shows the means, SDs, and CVs of the tHcy values and the number of values obtained at each of the five tHcy concentrations. There were no significant differences among the mean tHcy values assayed by the six different methods except at the lowest and highest tHcy concentrations (pools A and I, respectively). At 33 μmol/L tHcy, values obtained by the FPIA method were significantly lower ($P = 0.029$) compared with values obtained by HPLC-FD with TBP/SBDF as the reduction/derivatization agents.

Within-method variation was again lowest for the FPIA assay; the mean CV was 4.4% compared with mean CVs >10% for the HPLC methods. The mean among-method variation for 170 samples was 13%. With regard to differences in CV as a function of tHcy concentration, the FPIA method had a larger CV (7.4%) at the lowest tHcy concentration compared with CVs obtained at increasing tHcy concentrations; the amino acid analyzer method had the largest CVs (17% and 16%) at the lowest and highest tHcy concentrations, respectively. The HPLC methods showed no difference in CVs as a function of tHcy concentration.

To confirm the observation that tHcy values obtained by the FPIA method were lower at increased tHcy concentrations compared with values obtained by HPLC-FD (TBP/SBDF), we reanalyzed each of the 10 pools by both methods over 10 days. As shown in Table 3, the mean differences in tHcy concentrations reached significance for 8 of 10 pools. Moreover, differences became more significant as the tHcy concentration increased. Intralaboratory imprecision was lower for the FPIA method (CV, 3.8%) compared with the HPLC method (CV, 6.7%).

Discussion

Reports that moderate hyperhomocysteinemia is associated with CAD *(1–7)* and venous thrombosis *(8–10)* have prompted clinicians to include analysis of tHcy as part of the risk assessment profile for vascular diseases. Al-

Table 1. Types of methodologies used and within- and among-method variation.

Method	Reduction/ Derivatization agents	n	Mean ± SD of respective pools, μmol/L (CV, %)					Mean CV, %
Survey 1			**A**	**B**	**J**	**G**	**D**	
Within method								
HPLC-FD	$NaBH_4$/MBrB	3	5.47 ± 1.83 (33)	8.27 ± 1.00 (12)	13.70 ± 1.23 (9.0)	23.03 ± 3.38 (15)	44.97 ± 2.05 (4.6)	15
HPLC-FD	TBP/SBDF	2	6.20 ± 0.57 (9.2)	9.05 ± 0.64 (7.1)	15.40 ± 1.13 (7.3)	25.70 ± 0.99 (3.8)	47.85 ± 3.18 (6.6)	6.8
HPLC-FD	TCEP/SBDF	3	6.93 ± 1.07 (15)	9.97 ± 1.19 (12)	16.67 ± 2.36 (14)	29.07 ± 6.43 (22)	53.63 ± 12.11 (23)	17
HPLC-ED	$NaBH_4$/−[a]	2	5.25 ± 0.35 (6.7)	9.05 ± 0.49 (5.4)	15.40 ± 2.97 (19)	29.80 ± 11.46 (38)	53.75 ± 20.86 (39)	22
AA[b] analyzer	DTT/Ninhydrin	1	3.9	7.4	12.0	19.3	36.3	
Among methods		11	5.82 ± 1.35 (23)	8.94 ± 1.12 (12)	14.97 ± 2.17 (14)	26.05 ± 6.05 (23)	48.66 ± 10.34 (21)	19
Survey 2			**A**[c]	**G**	**F**	**H**	I	
Within method								
HPLC-FD	$NaBH_4$/MBrB	3	5.83 ± 0.85 (15)	11.23 ± 0.64 (5.7)	15.67 ± 0.81 (5.2)	17.57 ± 0.74 (4.2)	31.23 ± 2.89 (9.3)	7.8
HPLC-FD	TBP/SBDF	5	5.48 ± 0.79 (14)	11.84 ± 1.89 (16)	16.56 ± 2.58 (16)	19.10 ± 4.28 (22)	35.98 ± 5.56 (16)	17
HPLC-FD	TCEP/SBDF	3	6.37 ± 1.21 (19)	12.17 ± 1.63 (13)	16.93 ± 1.59 (9.4)	19.53 ± 1.85 (9.5)	32.63 ± 3.50 (11)	12
HPLC-ED	$NaBH_4$/−[a]	3	5.57 ± 0.55 (9.9)	11.17 ± 0.55 (4.9)	16.13 ± 0.95 (5.9)	19.27 ± 1.63 (8.4)	32.60 ± 4.29 (13)	8.5
AA analyzer	DTT/ninhydrin	2	4.75 ± 0.78 (3.5)	10.40 ± 1.84 (18)	17.30 ± 0.57 (3.3)	18.15 ± 0.07 (0.4)	29.90 ± 6.65 (22)	9.4
FPIA	DTT/−[a]	7	5.51 ± 0.41 (7.4)	10.94 ± 0.66 (6.0)	15.57 ± 0.91 (5.8)	18.20 ± 1.02 (5.6)	32.10 ± 1.54 (4.8)	5.9
Among methods		23	5.62 ± 0.77 (14)	11.33 ± 1.26 (11)	16.22 ± 1.51 (9.3)	18.64 ± 2.21 (12)	32.83 ± 4.00 (12)	12
Survey 3			**E**[c]	**B**[c]	**H**[c]	**C**[c]	**I**[c]	
Within-method								
HPLC-FD	$NaBH_4$/MBrB	1	9.3	11.6	23.8	30.7	43.1	
HPLC-FD	TBP/SBDF	1	8.3	9.9	20.7	25.5	37.0	
HPLC-FD	TCEP/SBDF	5	7.36 ± 0.50 (6.7)	8.84 ± 1.01 (11)	18.64 ± 1.75 (9.4)	24.08 ± 1.59 (6.6)	34.84 ± 2.66 (7.6)	8.4
HPLC-ED	$NaBH_4$/−[a]	2	7.10 ± 0.00 (0.0)	9.15 ± 0.49 (5.4)	20.00 ± 0.28 (1.4)	24.70 ± 1.70 (6.9)	34.25 ± 1.63 (4.8)	3.7
AA analyzer	DTT/ninhydrin	1	7.5	8.0	16.4	21.4	31.6	
FPIA	DTT/−[a]	6	7.10 ± 0.17 (2.4)	8.68 ± 0.40 (4.6)	18.22 ± 0.46 (2.5)	22.85 ± 0.47 (2.1)	32.23 ± 0.60 (1.9)	2.7
LC-MS/MS	DTT/−[a]	1	7.1	9.0	19.5	24.3	35.5	
Among methods		17	7.40 ± 0.63 (8.5)	9.01 ± 0.98 (11)	18.96 ± 1.87 (9.9)	24.03 ± 2.27 (9.4)	34.24 ± 3.18 (9.3)	9.6

[a] No derivatization agent used.

[b] AA, amino acid; DTT, dithiothreitol.

[c] $P < 0.05$ for mean tHcy values obtained in: survey 2 (pool A) by HPLC-FD (TCEP/SBDF) vs amino acid analyzer; survey 3 (pools E, B, H, C, and I) for HPLC-FD ($NaBH_4$/MBrB) vs HPLC-FD (TCEP/SBDF), HPLC-ED, amino acid analyzer, FPIA, and LC-MS/MS (among methods); pools C and I for HPLC-FD ($NaBH_4$/MBrB) vs HPLC-FD (TBP/SBDF); pool E for HPLC-FD (TBP/SBDF) vs HPLC-FD (TCEP/SBDF), HPLC-ED, and FPIA; HPLC-FD (TCEP/SBDF) vs HPLC-ED; pools C and H for amino acid analyzer vs HPLC-FD (TBP/SBDF) and HPLC-ED; pool I, FPIA vs HPCL-FD (TBP/SBDF) and HPLC-FD (TCEP/SBDF).

Table 2. Within- and among-method variation in three surveys combined.

Method	Reduction/ Derivatization agents	Mean ± SD, μmol/L (CV, %)					Mean CV, %
Within method		A[a]	B	H	C	I[a]	
HPLC-FD	$NaBH_4$/MBrB	5.65 ± 1.29 (23) (n = 6)	9.10 ± 1.86 (20) (n = 4)	19.13 ± 3.17 (17) (n = 4)	24.95 ± 4.73 (19) (n = 4)	34.20 ± 6.38 (19) (n = 4)	20
HPLC-FD	TBP/SBDF	5.69 ± 0.77 (14) (n = 7)	9.33 ± 0.67 (7.2) (n = 3)	19.37 ± 3.88 (20) (n = 6)	25.63 ± 0.71 (2.8) (n = 3)	36.15 ± 4.99 (14) (n = 6)	12
HPLC-FD	TCEP-SBDF	6.65 ± 1.07 (16) (n = 6)	9.26 ± 1.15 (12) (n = 8)	18.98 ± 1.71 (9.0) (n = 8)	25.95 ± 4.46 (17) (n = 8)	34.01 ± 2.97 (8.7) (n = 8)	13
HPLC-ED	$NaBH_4$/−[b]	5.44 ± 0.46 (8.4) (n = 5)	9.10 ± 0.41 (4.5) (n = 4)	19.56 ± 1.23 (6.3) (n = 5)	27.25 ± 7.31 (27) (n = 4)	33.26 ± 3.27 (9.8) (n = 5)	11
AA[c] analyzer	DTT/ninhydrin	4.47 ± 0.74 (17) (n = 3)	7.70 ± 0.42 (5.4) (n = 2)	17.57 ± 1.01 (5.8) (n = 3)	20.35 ± 1.49 (7.3) (n = 2)	30.47 ± 4.80 (16) (n = 3)	10
FPIA	DTT/−[b]	5.51 ± 0.41 (7.4) (n = 7)	8.68 ± 0.40 (4.6) (n = 6)	18.21 ± 0.78 (4.3) (n = 13)	22.85 ± 0.47 (2.1) (n = 6)	32.16 ± 1.15 (3.6) (n = 13)	4.4 4.4
LC-MS/MS	DTT/−[b]	n = 0	9.0 (n = 1)	19.5 (n = 1)	24.3 (n = 1)	35.5 (n = 1)	
Among methods		5.67 ± 0.97 (17) (n = 34)	8.98 ± 1.0 (11) (n = 28)	18.78 ± 2.00 (11) (n = 40)	24.83 ± 4.17 (17) (n = 28)	33.43 ± 3.63 (11) (n = 40)	13 13

[a] $P < 0.05$ for mean tHcy values obtained for: pool A, HPLC-FD (TBP/SBDF) vs amino acid analyzer; HPLC-FD (TCEP/SBDF) vs HPLC-ED, amino acid analyzer, and FPIA (among methods); amino acid analyzer vs among-method mean; pool I, by HPLC-FD (TBP/SBDF) vs amino acid analyzer and FPIA.

[b] No derivatization agent used.

[c] AA, amino acid; DTT, dithiothreitol.

though there is increased use of tHcy measurements in a clinical setting and a need to interpret reference intervals *(22)*, few large-scale studies have assessed the methodologic differences, interlaboratory variation, and analytical imprecision of the various methods available for measurement of plasma tHcy, especially at the increased tHcy concentrations attained after methionine loading. In most previous studies, samples were modified by adding either D,L-homocysteine or L-homocystine to plasma. Adding D,L-homocysteine has the major disadvantage in that the D-form is not present physiologically. In addition, there will be a lack of correlation between chromatographic and immunologic methods because the enzyme used in the initial step of the immunologic methods does not react with D-homocysteine. Addition of the L-form will produce a tHcy distribution in plasma that is different from that found in physiologic samples, in which >70% of the tHcy is bound to protein and the remaining exists as L-homocystine or as a mixed disulfide with cysteine *(23)*. Samples obtained after a methionine load are similar to those obtained after a meal with high protein content and thus should reflect the normal physiologic distribution of the different forms of tHcy. A major strength of the current study is the use of physiologic samples with high tHcy concentrations obtained after methionine loading, thus obviating the need to add endogenous compounds to plasma.

Table 3. Comparison of mean tHcy concentrations analyzed by HPLC-FD (TBP/SBDF as reduction/derivatization agents) and FPIA methods on 10 pools assayed over 10 days.

Pool	Mean ± SD, μmol/L (CV, %) HPLC-FD (TBP/SBDF)	FPIA	*P*
A	6.0 ± 0.47 (7.8)	6.0 ± 0.28 (4.7)	0.834
E	8.0 ± 0.61 (7.6)	7.4 ± 0.21 (2.8)	0.016
B	9.3 ± 0.75 (8.1)	9.1 ± 0.46 (5.1)	0.479
G	12.6 ± 0.70 (5.6)	11.6 ± 0.46 (4.0)	0.001
J	15.5 ± 0.94 (6.1)	14.2 ± 0.27 (1.9)	0.001
F	17.7 ± 1.70 (9.6)	16.0 ± 0.41 (2.6)	0.005
H	21.4 ± 0.87 (4.1)	19.0 ± 0.91 (4.9)	<0.001
C	26.9 ± 1.64 (6.1)	23.6 ± 0.88 (3.7)	<0.001
I	38.3 ± 2.58 (6.7)	32.8 ± 1.57 (4.8)	<0.001
D	48.6 ± 2.77 (5.7)	41.5 ± 1.56 (3.8)	<0.001
Overall	(6.7)	(3.8)	

Previous studies from our laboratory have shown that within-person and intralaboratory methodologic variances for both fasting and PML tHcy were relatively low, giving a high reliability coefficient for homocysteine *(21, 24)*. Thus, the methods used for measuring tHcy appear to have a high degree of analytical precision. For minimum performance of a method, it has been suggested that analytical imprecision should be $<0.75\ CV_{within\text{-}subject}$ *(25)*. We have shown that intraindividual variability ($CV_{within\text{-}subject}$) for fasting and PML tHcy varies 2.2% to 7.0% over 1 month *(21, 24)*, which suggests that analytical imprecision needs to be <5.3%. Our analysis of tHcy values obtained on five pools analyzed in two of three surveys showed that only the FPIA method (CV, 4.4%) meets this performance goal. The low imprecision of the FPIA method compared with HPLC methods is consistent with the findings of others *(14, 17–19, 26)*. The mean among-method CV of 13% for 170 samples assayed over

18 months is higher than the mean among-laboratory CVs of 9.2% (n = 46) and 9% (n = 9) reported by Pfeiffer et al. *(18)* and Møller et al. *(16)*, respectively. The higher among-method CV in our study can probably be attributed to the fact that we analyzed a larger number of samples over a longer period of time and at higher concentrations of endogenous tHcy.

A universally accepted reference material for homocysteine is not yet available; therefore, in this study each laboratory used its own calibrator. Tripodi et al. *(19)*, in a multicenter study of homocysteine measurement, showed that variation between laboratories ranged from 13.9% to 15.6%; however, CV values were <5.2% when a common plasma standard was used for calculation of tHcy concentrations. Studies have also shown that a given HPLC method, using a single standard as calibrator, is precise. Ubbink et al. *(20)*, using TBP/SBDF as reduction/derivatization agents, obtained within- and between-run CVs of 3.9–6.6%. Another study using simultaneous reduction of disulfide bonds with $NaBH_4$ and derivatization of sulfhydryl groups with MBrB reported intra- and interassay CVs of 3.31% and 4.85%, respectively *(27)*. A HPLC assay with internal standardization that used TCEP as reduction agent and SBDF to derivatize thiols had intraassay imprecision between 1.1% and 1.8% and interassay imprecision between 4.4% and 6.7% *(28)*. CVs in this same study were only slightly lower with the FPIA method on the Abbott IMx analyzer: within-run CVs were between 1.0% and 1.5%, and between-run CVs ranged from 2.5% to 4.9% *(29)*. In our laboratory, intralaboratory precision was also somewhat lower for the FPIA method (CV, 3.8%) compared with the HPLC-FD (TBP/SBDF) method (CV, 6.7%). Although HPLC methods may be to some degree inherently less precise than the FPIA method, the lower interlaboratory CVs obtained by FPIA in surveys 2 and 3 may also in part be attributable to the fact that calibrators are available with the commercial Abbott IMx Homocysteine Assay *(13)*.

In the absence of target values for the samples analyzed, we determined methodologic differences by comparing the mean tHcy concentrations obtained by each method to the mean concentrations obtained by other methods and to the among-method means. Because of the small number of laboratories performing each method in each of the three surveys and the large SDs of most methods, it is difficult to confirm that any differences in mean tHcy values are method specific. However, when the results of the three surveys were combined to obtain a larger sample number, it is of interest to note that for tHcy concentrations >30 μmol/L, values obtained by the FPIA method were significantly lower than values obtained by HPLC-FD using TBP/SBDF as the reduction/derivatization agents. We confirmed this finding in our laboratory by reanalyzing the 10 pools over 10 different days by each of the two methods. The mean value that we obtained for each pool by FPIA was slightly higher than the values reported in the survey; nevertheless, our mean values by FPIA were still significantly lower than those obtained by HPLC-FD using TBP/SBDF. Whether this difference in values between the two methods is attributable to methodologic differences or originates from the calibrators for each assay is uncertain.

Because clinical studies have associated ranges of tHcy concentrations with the risk for CAD, our observation that results from the FPIA method were significantly different from those obtained by the HPLC-FD (TBP/SBDF) method warrants further investigation into the accuracy of tHcy measurements. Currently, there is an intense effort by the IFCC Working Group on Standardization of Total Plasma Homocysteine Measurements, which recently met at the 3rd International Conference on Homocysteine Metabolism, to standardize plasma tHcy assays. This includes definition of a true homocysteine standard, establishing reference methods, and the use of external quality assessment programs to further monitor analytical imprecision, decrease analytical differences, and standardize tHcy measurements among laboratories.

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