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Detection of moderate hyperhomocysteinemia: Comparison of the Abbott fluorescence polarization immunoassay with the Bio-Rad and SBD-F high-performance liquid chromatographic assays

Short Communication

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Summary. The importance of accurate methods for homocysteine measurement has been emphasized. We compared the results obtained with the most commonly used high-performance liquid chromatography (HPLC) assay, and two recently commercially available methods: another HPLC and a fluorescence polarization immunoassay, in plasmas from normo- or hyperhomocysteinemic patients. A significant agreement between the different methods in classifying the results as hyper or normal-homocysteinemia was observed. However, a significant difference between the results was found. Standardization is urgently necessary to improve the concordance of homocysteine determination.

Keywords: Amino acids – Homocysteine – High-performance liquid chromatography – Fluorescence polarization immunoassay

Introduction

Multiple studies have shown that a moderately elevated plasma homocysteine concentration is an independent risk factor for atherothrombotic vascular disease (Welch and Loscalzo, 1998). The importance of accurate methods for mild elevations in homocysteine measurement has been emphasized. A high performance liquid chromatography (HPLC) technique with fluorescence detection and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) derivatization described by Ubbink et al. (1991) is the most commonly used in epidemiological studies. New methods have recently been commercially

available: another HPLC technique (Bio-Rad HPLC) (Dias et al., 1998) and a fluorescence polarization immunoassay (FPIA) (Shipchandler and Moore, 1995; Leino, 1999). However the data lack with comparison of homocysteine determination by these three different methods.

The aim of the study was to compare the results obtained with the SBD-F HPLC technique, the FPIA and the Bio-Rad HPLC methods and to examine the agreement between the different methods in classifying the results as hyper or normal-homocysteinemia, in order to choice the technique(s) which are the most appropriate for interlaboratory studies.

Materials and methods

Subjects

Plasmas were obtained from normo- or hyper-homocysteinemic fasting or post-methionine load patients for recent studies concerning homocysteine metabolism (9 patients with hyper- or hypo-thyroidism, 11 patients with coronary lesions, 12 without coronary lesions, 5 patients with diabetes mellitus). The study protocols were approved by the local ethical committee.

Blood samples were immediately placed on ice and centrifuged within one hour after the collection. EDTA-Plasmas were frozen and stored at −20°C until analysis within the next year.

Laboratory procedures

Total homocysteine was routinely determined using the SBD-F HPLC method (Ubbink et al., 1991). Briefly, after reduction with tri-n-butylphosphine, deproteinization with trichloroacetic acid, centrifugation and derivatization with SBD-F, the compounds are isocratically separated by C18 reversed-phase HPLC and quantified by fluorescence detection.

The Abbott fluorescence polarization immunoassay (Shipchandler and Moore, 1995), using an IMx analyzer, involves dithiothreitol reduction, enzyme conversion of homocysteine to S-adenosyl-L-homocysteine and quantification with specific monoclonal antibody and fluoresceinated analog tracer.

The Bio-Rad method (Dias et al., 1998) involves trialkylphosphine reduction, derivatization with 4-(Aminosulfonyl)-7-fluorobenzo-2-oxa-1,3-diazole and deproteinization with trichloroacetic acid. The homocysteine adduct is then isocratically separated by a C18 reversed-phase HPLC cartridge and quantified by fluorescence detection.

The SBD-F HPLC, FPIA and Bio-Rad HPLC assays were calibrated against calibrators prepared from crystalline D, L-homocysteine (Sigma Chemicals) or supplied by the respective manufacturers: a S-adenosyl-L-homocysteine solution and a serum calibrator, respectively.

The within-run precision for the assays (SBD-F HPLC, FPIA and Bio-Rad HPLC), determined by replicate analysis (n = 4 − 11) of plasma pools with homocysteine levels of 12 μmol/L, gave satisfactory and equal results (coefficients of variation 3%, 3.3%, and 2.5% respectively).

Normal ranges were 7.5–16.4 μmol/L for the SBD-F HPLC technique as determined in our laboratory (n = 149 normal subjects, 90% confidence interval (Nicolas and Chango, 1997)) and 4.45–12.42 μmol/L, $<$15 μmol/L for the FPIA and Bio-Rad HPLC technique as determined by the respective manufactures, respectively.

Statistical analysis

Comparison of the results was performed using Bland and Altman difference plots (Bland, 1986) and by Student's *t* test for paired series. The 95% confidence interval for the mean difference is given by the mean ± 2XSEM (standard error of the mean). A *p* value of less than 0.05 was considered as significant.

Results of each technique were classified into two classes (low or normal and high) according to their respective normal ranges. Agreement for the classification of the results as hyperhomocysteinemia or not was determined by the κ measure of reliability. A z value >1.96 was considered as significant.

Results and discussion

A significant agreement between the classification of the results as hyper or normal-homocysteinemia obtained by each technique and its respective normal range was observed. κ values were 0.68 ($z = 5.12$ and 3.67) with 15% of discrepancies for the comparison of the classes for SBD-F HPLC vs. FPIA, and Bio-Rad HPLC vs. FPIA, respectively. For SBD-F HPLC vs. Bio-Rad HPLC, a better agreement was obtained: κ value was 0.85 ($z = 4.71$) with 7% of discrepancies.

The comparison of the results demonstrated no agreement between the three assays. None of the methods should be used interchangeably. Means of total homocysteine determined by the three techniques were arranged in the order: SBD-F HPLC < FPIA < Bio-Rad HPLC (Table 1a). The comparison of the SBD-F HPLC and FPIA methods showed the widest scatter of differences (Fig. 1a) and the lowest absolute mean difference (Table 1b). Both HPLC methods displayed a low scatter of difference data points and presented with the lowest differences with increasing concentrations and the widest mean proportional bias (Table 1c, Fig. 1b). A significant difference between the HPLC values was observed (Table 1a, c), but the correlation coefficient was good ($R = 0.981$, $p < 0.0001$). For homocysteine concentrations $<20\mu$mol/L, the Bio-Rad HPLC and FPIA results presented with low differences and mean proportional bias. Larger differences were observed with increasing homocysteine values (Fig. 1c). Our comparison with SBD-F HPLC showed larger discrepancies than those obtained with gas chromatography-mass spectrometry (GC-MS) (Pfeiffer et al., 1999; Ubbink et al., 1999).

A closer agreement between the methods for fasting homocysteine concentrations has been observed with GC-MS and FPIA than GC-MS and SBD-F HPLC (Ubbink, 1999). Others demonstrated that, when compared with GC-MS, SBD-F HPLC and FPIA showed virtually no apparent bias and narrow limits of agreement (Pfeiffer et al., 1999). However, the within-method variation was reported to be high (9.8%) for 3 laboratories with SBD-F HPLC methods (Pfeiffer et al., 1999).

Compared with the SBD-F HPLC method, the FPIA and Bio-Rad HPLC methods are rapid, plasma sparring, commercially available and easier to perform. The FPIA assay is the easiest method and is appropriate for

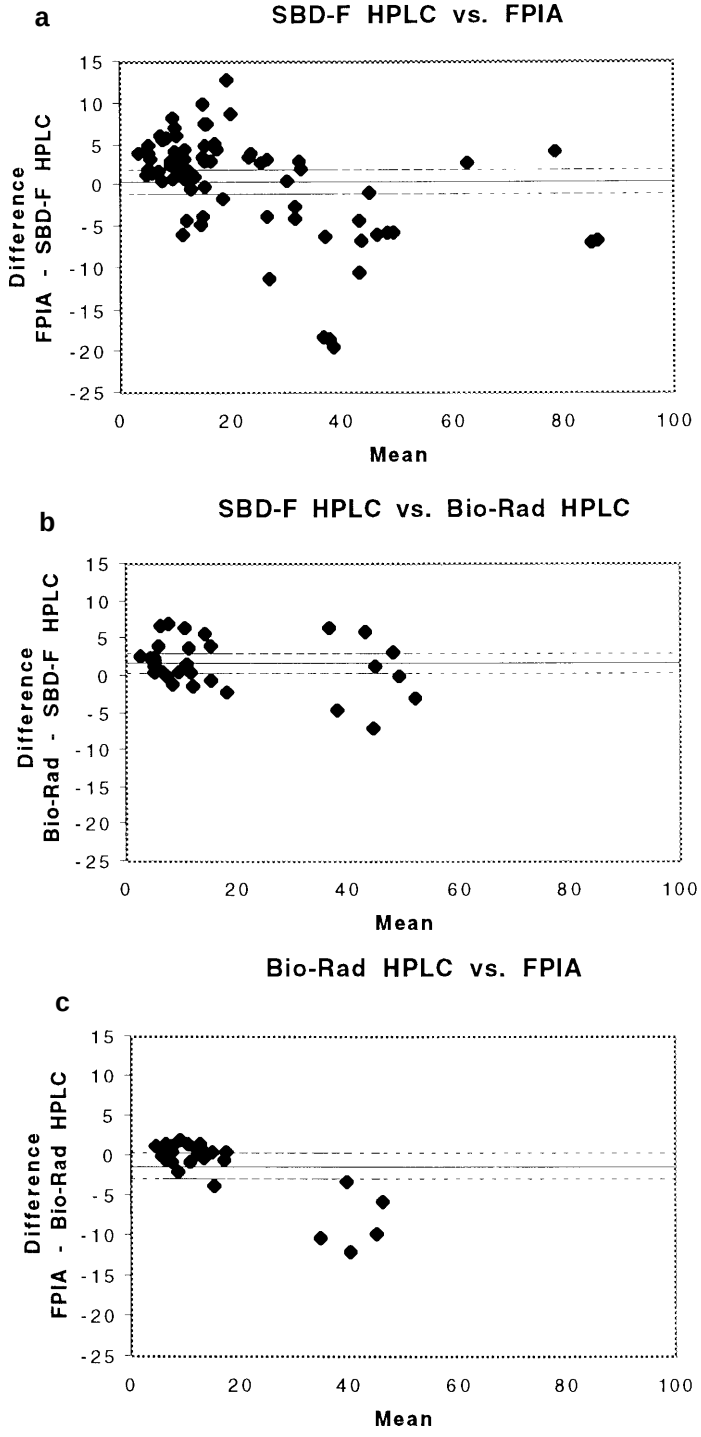

Fig. 1a–c. Scatter plots showing the differences between SBD-F* HPLC, FPIA* and Bio-Rad HPLC technics for homocysteine determination in EDTA-plasma samples from patients with normo- or hyper-homocysteinemia. Homocysteine concentrations are expressed in μmol/L. Dashed lines show the central 0.95 confidence interval of mean. *SBD-F* ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. *FPIA* fluorescence polarization immunoassay

Table 1a–c. Comparison of the results obtained for plasma homocysteine (expressed in μmol/L) determined with the 3 methods: SBD-F HPLC vs. FPIA and Bio-Rad HPLC, SBD-F HPLC vs. FPIA, SBD-F HPLC vs. Bio-Rad HPLC and Bio-Rad HPLC vs. FPIA

	Mean	SD	Minimum	Maximum	95% confidence interval of mean
	a. SBD-F HPLC vs. FPIA and Bio-Rad HPLC (n = 26)				
SBDF-HPLC*	14.91	15.11	1.30	49.20	[8.99; 20.83]
FPIA	15.53	11.39	5.20	43.30	[11.07; 19.99]
Bio-Rad HPLC*	16.92	14.67	3.90	49.93	[11.16; 22.68]
	b. SBD-F HPLC vs. FPIA (n = 70)				
SBDF-HPLC	22.54	20.36	1.30	89.70	[17.68; 27.40]
FPIA	22.90	17.40	5.20	82.90	[18.74; 27.06]
Difference FPIA – SBD-F HPLC	0.35	6.33	−19.49	12.72	[−1.16; 1.86]
	c. SBD-F HPLC vs. Bio-Rad HPLC (n = 30)				
SBDF-HPLC*	17.67	17.23	1.30	53.80	[11.36; 23.96]
Bio-Rad HPLC*	19.27	16.26	3.90	50.80	[13.33; 25.21]
Difference FPIA – SBD-F HPLC	1.60	3.40	−7.10	6.90	[0.36; 2.84]
	d. Bio-Rad HPLC vs. FPIA (n = 26)				
Bio-Rad HPLC	16.92	14.67	3.90	49.93	[11.16; 22.68]
FPIA	15.53	11.39	5.20	43.30	[11.07; 19.99]
Difference FPIA – Bio-Rad HPLC	−1.39	3.89	−12.04	1.90	[−2.91; 0.13]

*Results obtained with the SBD-F HPLC and Bio-Rad HPLC techniques are significantly different ($p < 0.05$).

interlaboratory analysis of large series of samples. However, its wide use is limited by its high cost. The Bio-Rad HPLC method is also attractive as it is simple and cheaper and is concordant with FPIA for the detection of moderate hyperhomocysteinemia. The Bio-Rad assay presents the closest agreement with our actual technique.

Different methodologies, calibration materials and reduction of homocysteine-containing complex could account for the observed differences. Standardization and inter-method comparison with routine assays and/or frequent quality assessment programs are urgently necessary to improve the concordance of homocysteine determination.

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